

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number
WO 01/16603 A2

- (51) International Patent Classification⁷: G01N 33/68 (74) Agents: KIDDLE, Simon, J. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).
- (21) International Application Number: PCT/GB00/03277
- (22) International Filing Date: 24 August 2000 (24.08.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9920745.8 2 September 1999 (02.09.1999) GB
0002975.1 9 February 2000 (09.02.2000) GB
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): PROTEOME SCIENCES PLC [GB/GB]; Coveham House, Downside Bridge Road, Cobham, Surrey KT11 3EP (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): CAWTHORNE, Michael [GB/GB]; The University of Buckingham, Yeomanry House, Hunter Street, Buckingham MK18 1EG (GB). SANCHEZ, Jean-Charles [CH/CH]; Frank-Thomas 42, CH-1208 Geneva (CH).

Published:

— Without international search report and to be republished upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/16603 A2

(54) Title: METHODS AND COMPOSITIONS RELATING TO BODY WEIGHT AND EATING DISORDERS

(57) Abstract: Methods and compositions relating to body weight and eating disorders, such as obesity, are disclosed. Specifically, proteins that are differentially expressed in these conditions are identified. In one aspect, the invention provides a method of screening an agent to determine its usefulness in treating a condition associated with regulation of appetite and body weight, the method comprising: (a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of body weight or eating dysfunction; (b) obtaining a sample of relevant tissue taken from, or representative of, a subject having body weight or eating disorders, who or which has been treated with the agent being screened; (c) determining the presence, absence or degree or expression of the differentially expressed protein or proteins in the tissue from, or representative of, the treated subjects; and (d) selecting or rejecting the agent according to the extent to which it changes the expression of the differentially expressed protein or proteins in the treated subject having body weight or eating disorders.

Methods and Compositions Relating to Body Weight and
Eating Disorders

Field of the Invention

5 The present invention relates to methods and compositions
relating to body weight disorders and eating disorders in
conditions such as obesity. Specifically, the present
invention identifies and describes proteins that are
10 differentially expressed in body weight and/or eating
disordered states relative to their expression in normal
or non-body weight disordered states and in particular
identifies and describes proteins associated with the
regulation of appetite and body weight. Further, the
present invention identifies and describes proteins via
15 their ability to interact with gene products involved in
the regulation of body weight and appetite. Still
further, the present invention provides methods,
particularly experimental paradigms for the
identification of differentially expressed proteins that
20 are potential molecular targets for compounds to treat
body weight and/or eating disorders including, but not
limited to, obesity. Still further, the present
invention provides methods for the identification and
therapeutic use of compounds for the treatment of body
25 weight and/or eating disorders including, but not limited
to, obesity.

Background of the Invention

30 Body weight disorders, including eating disorders, are
one of the major public health problems in all
industrialised countries and is a growing problem in
countries undergoing rapid acculturalisation. Obesity,
the most prevalent of eating disorders, is the most
common nutritional disorder in the Western world and can

- have a prevalence of up to 50% in middle-aged and elderly populations. However, it is also an increasing problem in children. Other weight disorders, such as anorexia nervosa and bulimia nervosa are also serious health risks and affect approximately 0.2% of the population, particularly female, in Western countries. Anorexia and cachexia are major features of other diseases such as cancer, AIDS and trauma.
- Obesity, defined as an excess body fat relative to lean body mass, also contributes to many other diseases. For example, obesity is responsible for the increased incidence of diseases such as coronary artery disease, stroke and non-insulin dependent diabetes. Obesity is not merely a behavioural problem, i.e. the result of voluntary hyperphagia. Rather, the differential body composition observed between obese and non-obese subjects results from differences in both metabolism and neurological/metabolic interactions. These differences are to some extent genetically inherited but the nature of the gene products that control body weight and body composition are unknown. Attempts to identify protein molecules involved in the control of body weight have been largely empiric and the nature of the mechanisms by which body composition and/or substrate flux are monitored have not yet been identified.

The epidemiology of obesity strongly shows that the disorder exhibits inherited characteristics (Stunkard, 1990, N. Eng. J. Med. 322: 1483). Moll et al., have reported that, in many populations, obesity seems to be controlled by a few genetic loci (Moll et al., 1991, Am. J. Hum. Gen. 49: 1243). In addition human twin studies

strongly suggest a substantial genetic basis in the control of body weight, with estimates of heritability of 80-90% (Simopoulos, A.P. & Childs, B., eds., 1989, in "Genetic Variation and Nutrition in Obesity", World
5 Review of Nutrition and Diabetes 63, S. Karger, Basel, Switzerland; Borjeson, M., 1976, Acta. Paediatr. Scand., 65: 279-287).

10 Further, studies of non-obese persons who deliberately attempted to gain weight by systematically over-eating found that some were more resistant to such weight gain than others and were able to maintain an elevated weight only by very high caloric intake. In contrast,
15 spontaneously obese individuals are able to maintain their obese status with normal or only moderately elevated caloric intake.

In addition, it is a commonplace experience in animal husbandry that different strains of swine, cattle, etc.,
20 have different predispositions to obesity. Studies of the genetics of human obesity and of models of animal obesity demonstrate that obesity results from complex defective regulation of both food intake, food induced energy expenditure and of the balance between lipid and
25 lean body anabolism.

There are a number of genetic diseases in man and other species, which feature obesity among their more prominent symptoms, along with, frequently, dysmorphic features and
30 mental retardation. Although no mammalian gene associated with an obesity syndrome has yet been characterised in molecular terms, a number of such diseases exist in humans. For example, Prader-Willi

syndrome (PWS) affects approximately 1 in 20,000 live births, and involves poor neonatal muscle tone, facial and genital deformities, and generally obesity. The genetics of PWS are very complex, involving, for example, genetic imprinting, in which development of the disease seems to depend upon which parent contributes the abnormal PWS allele. In approximately half of all PWS patients, however, a deletion on the long arm of chromosome 11 is visible, making the imprinting aspect of the disease difficult to reconcile. Given the various symptoms generated, it seems likely that the PWS gene product may be required to normal brain function, and may, therefore, not be directly involved in adipose tissue metabolism.

In addition to PWS, many other pleiotropic syndromes, which include obesity as a symptom, have been characterised. These syndromes are more genetically straightforward, and appear to involve autosomal recessive alleles. The diseases, which include, among others, Ahlstrom, Carpenter, Bardet-Biedl, Cohen, and Morgagni-Stewart-Monell Syndromes. However, each of these is rare and they do not account for the human obesity epidemic.

There are a number of animal models with mutations that are associated with body weight and body composition disorders and attempts have been made to utilise such animals as models for the study of obesity. The best studied animal models for genetic obesity are mice, which contain the autosomal recessive mutations ob/ob (obese) or db/db (diabetes). These mutations are on chromosome 6 and four respectively, but lead to clinically similar

pictures provided the genes are expressed on the same background strain. The ob gene product has been identified as 16kDa polypeptide produced primarily by adipose tissue that provides a signal to the brain on the adipose tissue fat stores. Mice with a mutation, resulting in no circulating protein (called leptin) are hyperphagic, obese, have poor thermo-regulation and non-shivering thermogenesis and are insulin resistant with impaired glucose tolerance. Treatment of these mice with recombinant leptin reduces food intake and stimulates energy expenditure so that the mice become less obese.

The db/db mice have a mutation in the receptor for leptin so that normal signal transduction via the JAK/STAT pathway does not occur. This mutation, when on the C57BI/6 background, is phenotypically identical to the ob mutation, but causes additionally frank diabetes when on the C57BI/Ks background. Other single gene mutations in mice associated with obesity, include the yellow mutation at the agouti locus, mutations at the fat and tubby loci and an autosomal dominant mutation at the adipose locus on chromosome 7.

Other mutant animal models include fa/fa (fatty) rats and ZDF fatty rats, which bear strong respective similarities with the ob/ob and db/db mice. Thus the fa/fa rat is hyperphagic, obese, insulin resistant, very hyperinsulinaemic and glucose intolerant, whereas the ZDF rat is hyperphagic, obese, insulin resistant and hyperinsulinaemic, but develops frank diabetes after approximately 6 weeks of age.

Inbred mouse strains, such as the NZO mouse, the Japanese

KK mouse are models of obesity. Further, desert rodents, such as spiny mice and sand rats are neither insulin resistant nor diabetic in their natural habitats, but do become insulin resistant and glucose intolerant when fed on a standard laboratory diet.

Obesity is a common feature of elderly rodents and the development of obesity can be accelerated by feeding diets with a high fat content, whether these diets are synthetic homogenous diets or are the result of supplementation of replacement of the normal rat chow by human food with a high fat content (cafeteria diet).

In animal studies, it has been demonstrated that, as in humans, some animals are able to resist the obesity inducing effects of a diet with a high fat content. This resistance appears to be at, at least, two levels. First, there are differences in preferences for diet high in fat. Thus, some animals do not overeat when presented with a high fat diet, whereas others do. Secondly, animals show differences in their ability to resist obesity by activating energy wasting mechanisms.

Farm animal studies have clearly demonstrated that selective breeding can be undertaken for common traits such as food efficiency, growth rate, leanness. Similarly studies in mice have been undertaken to selectively breed for obesity and leanness so that over several generations of brother/sister matings colonies of mice are produced from a common parent stock that are significantly different in the level of fatness.

All of these animal models have been used from time to

time to evaluate new drugs that were potential treatments for body weight and eating disorders, including obesity. However, although individual changes in enzyme activities have been identified in some of the animal models and how this might be altered by a drug therapy, no systematic evaluation has been made of the differences in protein expression in the tissues of normal animals and the obese animals. It is these changes in protein expression that underlie the development of body weight and eating disorders, including obesity. It is the same changes in protein expression that are likely to be causative of obesity in humans and in companion animals such as dogs and cats. Given the severity and prevalence of obesity, there exists a great need for the systematic identification of the disease causing proteins, since modulation of the expression level of such proteins back to the level in non-obese state represents a means of treating the disease condition.

20 Summary of the Invention

Broadly, the present invention relates to methods and compositions for the treatment of body weight and eating disorders, including but not limited to, obesity. More specifically, the present invention identifies and describes proteins that are differentially expressed in body weight and/or eating disordered states relative to their expression in normal, or non-body weight disordered states and also identifies proteins that are differentially expressed in response to manipulations relevant to body weight regulation and/or control of food intake. Such differentially expressed proteins may represent 'target proteins' and/or fingerprint proteins. Further, the present invention identifies and describes

proteins termed pathway proteins via their ability to interact with proteins involved in the regulation of body weight and/or food intake. Pathway proteins may also exhibit target protein and/or fingerprint protein characteristics.

Accordingly, in the first aspect, the present invention provides a method of screening an agent to determine its usefulness in treating a condition characterised by body weight disorders and/or eating disorders, the method comprising:

- (a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects have differential levels of obesity;
- (b) obtaining a sample of relevant tissue taken from, or representative of, a subject have obesity, who or which has been treated with the agent being screened;
- (c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the tissue from, or representative of, the treated subject; and,
- (d) selecting or rejecting the agent according to the extent to which it changes the expression of the differentially expressed protein or proteins in the treated obese subject.

Typically, an agent is selected if it changes the expression of a differentially expressed protein towards that of a normal weight subject.

In a further aspect, the present invention provides a method for the identification of an agent or agents for

use in the treatment of body weight and/or eating disorders comprising the steps of:

5 (a) identification of experimental and/or clinical paradigms that exhibit differential levels of obesity or lean or nutrient intake status (e.g. fed v fasted) or macronutrient selection (e.g. fat-preferring v non-fat preferring);

10 (b) identification of differentially expressed proteins in tissues of animals or humans exhibiting differential levels of obesity or leanness or nutrient intake states or macronutrient selection;

15 (c) selecting an agent that converts the expression of differentially expressed proteins in tissues of animals or humans exhibiting body weight or eating disorder dysfunctional states to that in the normal state for use in the treatment of the body weight or eating disordered state.

20 In a further aspect, the present invention provides the use of an agent identified by the above method for the preparation of a medicament for the treatment of a condition characterised by body weight or eating disorders. These conditions include obesity, non-insulin dependent diabetes (type 2 diabetes), Cushing syndrome, 25 anorexia nervosa and bulimia.

30 In a further aspect, the present invention provides a method of treating a condition characterised by body weight or eating dysfunction in a patient, the method comprising administering a therapeutically or prophylactically effective amount of such an agent identified by the above method.

In a further aspect, the present invention provides a method of determining the nature or degree of body weight and/or eating dysfunction in a human or animal subject, the method comprising:

- 5 (a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having a body weight and/or eating disorder;
- (b) obtaining a sample of the tissue from the
10 subject;
- (c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the sample; and
- (d) relating the determination to the nature or
15 degree of the body weight or eating disorder by reference to a previous correlation between such a determination and clinical information.

Conveniently, the patient sample used in method can be a
20 tissue sample or body fluid sample or urine. This method allows the causes of body weight or eating disorder dysfunction of a patient to be correlated to different types to prophylactic or therapeutic treatment available in the art, thereby enhancing the likelihood of obtaining
25 a beneficial response in the patient to the therapy.

In a further aspect, the present invention provides a method of treatment by the use of an agent that will restore the expression of one or more differentially
30 expressed proteins in the body weight or eating disorders dysfunction state to that found in the normal state in order to maintain a reduced body weight (prevent weight gain) or to prevent the re-emergence of an eating

disorder.

In a further aspect, the present invention provides a method whereby the pattern or differentially expressed
5 proteins in a tissue sample or body fluid sample or urine of an individual with body weight and/or eating disorder dysfunction is used to predict the most appropriate and effective therapy to alleviate the dysfunction state and to monitor the success of that treatment.

10

In a further aspect, the present invention provides a protein which is differentially expressed in relevant tissue from, or representative of subjects having
15 differential levels of obesity or leanness or nutrient intake states or macronutrient selection and which is as obtainable by the method of two-dimensional gel electrophoresis carried out on said tissue or a protein-containing extract thereof, the method comprising:

- 20 (a) providing non-linear immobilised pH gradient (IPG) strips of acrylamide polymer 3mm x 180mm;
- (b) rehydrating the IPG strips in a cassette containing 25ml of an aqueous solution of urea (8M), 3-
25 [(cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of acids and bases of pH 3.5 to 10 (2% w/v) and a trace of Bromophenol Blue:
- (c) emptying the cassette of liquid, transferring the strips to an electrophoretic tray fitted with humid electrode wicks, electrodes and sample cups, covering the
30 strips and cups with low viscosity paraffin oil;
- (d) applying 200 micrograms of an aqueous solution of dried, powdered material of the relevant body tissue in urea (8M), CHAPS (4% w/v), Tris (40 mM), DTE (65mM),

SDS (0.05% w/v) and a trace of Bromophenol Blue to the sample cups, at the cathodic end of the IPG strips;

(e) carrying out isoelectric focusing on the gel at a voltage which increases linearly from 300 to 3500V during 3 hours, followed by another 3 hours at 3500V, and thereafter at 5000V for a time effective to enable the proteins to migrate in the strips to their pH-dependent final positions;

(f) equilibrating the strips within the tray with 100ml of an aqueous solution containing Tris-HCl (50mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 minutes;

(g) replacing this solution by 100ml of an aqueous solution containing Tris-HCl (50mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of Bromophenol Blue for 5 minutes;

(h) providing a vertical gradient slab gel 160 x 200 x 1.5mm of acrylamide/piperazine-diacrylyl cross-linker (9-16%T/2.6%C), polymerised in the presence of TEMED (0.5% w/v), ammonium persulphate (0.1% w/v) and sodium thiosulphate (5mM), in Tris-HCl (0.375M) pH 8.8 as leading buffer;

(i) over-layering the gel with sec-butanol for about 2 hours, removing the overlay and replacing it with water;

(j) cutting the IPG gel strips to a size suitable for the second dimensional electrophoresis, removing 6mm from the anode end and 14mm from the cathode end;

(k) over-laying the slab gel with an aqueous solution of agarose (0.5% w/v) and Tris-glycine-SDS (25mM-198mM - 0.1% w/v) as leading buffer, heated to 70°C and loading the IPG gel strips onto the slab gel through this over-layered solution;

- (l) running the second dimensional electrophoresis at a constant current of 40mA at 8-12°C for 5 hours; and,
- (m) washing the gel.

5 Examples of differentially expressed proteins are described in the examples below.

 Alternatively, fingerprint proteins may be used in methods for identifying compounds useful for the
10 treatment of body weight and/or eating disordered states. 'Target protein', as used herein, refers to a differentially expressed protein involved in body weight regulation and/or the control feeding such that modulation of the expression of that protein may act to
15 prevent or ameliorate the body weight and/or eating disordered state including, but not limited to, obesity.

 This invention is based, in part, on systematic search strategies involving modulations of body weight and/or
20 eating behaviours and obesity experimental paradigms, coupled with sensitive detection of proteins by 2D-electrophoresis.

 The invention further provides methods for the
25 identification of compounds that modulate the expression of proteins involved in body weight and feeding processes relevant to the regulation of energy balance. Still further, the present invention describes methods for the prevention and/or treatment of obesity, which may involve
30 the administration of such compounds to individuals predisposed to or exhibiting obesity.

 Additionally, the present invention describes methods for

the prognostic and diagnostic evaluation of subjects with body weight and eating disordered states in order to make a prognosis of the most effective therapy for each subject.

5

The examples presented below demonstrate the successful use of the experimental paradigms of the invention to identify target proteins associated with a body weight and/or eating disordered state.

10

Definitions

"Differential expression", as used herein, refers to at least one recognisable difference in tissue protein expression. It may be a quantitatively measurable, semi-quantitatively estimatable or qualitatively detectable difference in tissue protein expression. Thus, a differentially expressed protein (herein DEP) may be strongly expressed in tissue in the normal state and less strongly expressed or not expressed at all in tissue in the body weight or eating disordered state. Conversely, it may be strongly expressed in tissue in the disorder state but less strongly expressed or not expressed at all in the normal state. Similarly the differential expression can be either way around in the comparison between untreated and treated tissue. Further, expression may be regarded as differential if the protein undergoes any recognisable change between the two states under comparison.

30

Thus, in contrast to prior art methods as described in US Patent No:5,702,902 which identify differentially expressed genes by examining RNA, mRNA or cDNA libraries derived from different tissue types, the present

invention is based on methods which directly determine differentially expressed proteins present in tissue samples, by employing techniques such as 2D gel electrophoresis.

5

The term "paradigm" means a prototype example, test model or standard.

10

Wherever a differentially expressible protein is used in the screening procedure, it follows that there must have been at some time in the past a preliminary step of establishing a paradigm by which the differential expressibility of the protein was pre-determined. Once the paradigm has been established, it need not be re-

15 established on every occasion that a screening procedure is carried out. The term "establishing a paradigm" is to be construed accordingly.

20

Body weight and/or eating disordered state includes conditions in which the body mass is either below normal or above normal and conditions in which eating behaviour is abnormal. Conditions characterised by body weight and/or eating disordered state include obesity, anorexia, bulimia and cathexic states associated with cancer, AIDS

25 and trauma.

30

Nutrient intake status includes any paradigm providing a difference in nutrient intake such as differences between fed and fasting, over-feeding v normal feeding, meal eating v ad-libitum eating, sated v unsated.

Macronutrient selection includes paradigms in which individuals or animals select or are provided different

macronutrients such as high fat v low fat, fat preferring
v carbohydrate preferring.

5 "Relevant tissue" means any tissue which undergoes a
biological change in the body weight or eating disordered
state or the experimental paradigm.

10 "Tissue ... representative of ... subjects" means any tissue
in which the above-mentioned biological change can be
simulated for laboratory purposes and includes, for
example, a primary cell culture or cell line derived
ultimately from relevant tissue.

15 The term "subjects" includes human and animal subjects.

The treatments referred to above can comprise the
administration of one or more drugs or foodstuffs, and/or
other factors such as diet or exercise.

20 The differentially expressed proteins (DEPs) include
"fingerprint proteins", "target proteins" or "pathway
proteins".

25 The term "fingerprint protein", as used herein, means a
DEP, the expression of which can be used, alone or
together with other DEPs, to monitor or assess the
condition of a patient suspected of suffering from a
bodyweight or eating disordered state. Since these
proteins will normally be used in combination, especially
30 a combination of four or more, they are conveniently
termed "fingerprint proteins", without prejudice to the
possibility that on occasions they may be used singly or
along with only one or two other proteins for this

purpose. Such a fingerprint protein or proteins can be used, for example, to diagnose a particular type of body weight or eating disorder and thence to suggest a specific treatment for it.

5

The term "diagnosis", as used herein, includes the provision of any information concerning the existence, non-existence or probability of the disorder in a patient. It further includes the provision of information concerning the type or classification of the disorder or of symptoms, which are or may be experienced in connection with it. It encompasses prognosis of the medical course of the disorder.

10

The term "target protein", as used herein, means a DEP, the level or activity of which can be modulated by treatment to alleviate a body weight or eating disordered state. Modulation of the level or activity of the target protein in a patient may be achieved, for example, by administering the target protein, another protein or gene which interacts with it, or an agent which counteracts or reduces it, for example an antibody to the protein, competitive inhibitor of the protein or an agent which acts in the process of transcription or translation of the corresponding gene.

20

25

Alternatively or additionally, the DEPs can interact with at least one other protein or which a gene involved in the regulation of body weight or eating. Such other proteins are termed herein "pathway proteins" (PPs). The term is applied to the protein with which it, the DEP, interacts, not the DEP itself, although a pathway protein can be another DEP.

30

By way of example, embodiments of the present invention will now be described in more detail with reference to the accompanying figures.

5 Brief Description of the Figures

Figures 1A-E show computer images of stained 2-DGE gels from the liver tissue of lean mice identifying spots thereon, including DEPs.

10 Figures 2-8 show DEPs which are underexpressed in ob/ob mouse liver tissue relative to expression in lean mouse lean mouse liver tissue.

15 Figures 9-11 show DEPs which are underexpressed in ob/ob mouse skeletal muscle relative to expression in lean mouse skeletal muscle, and DEPs which are overexpressed in ob/ob skeletal muscle relative to lean mouse skeletal muscle.

20 Figures 12-23 show DEPs which are underexpressed in ob/ob mouse adipose tissue relative to lean mouse adipose tissue, and DEPs which are overexpressed in obese mouse adipose tissue relative to adipose tissue of lean mice.

25 Figures 24-29 show DEPs which are underexpressed in ob/ob mouse brown adipose tissue relative to lean mouse brown adipose tissue, and DEPs which are overexpressed in obese mouse brown adipose tissue relative to brown adipose tissue of lean mice.

30

Detailed Description

Methods and compositions for the treatment of body weight and/or eating disordered states including, but not

limited to, obesity. Proteins termed 'target proteins' and/or fingerprint proteins are described which are differentially expressed in body weight and/or eating disordered states relative to their expression in normal states and/or which are differentially expressed in response to manipulations relevant to the regulation of body weight and/or eating. Additionally, proteins termed 'pathway proteins' are described which interact with proteins involved in regulation of body weight and/or eating. Methods for the identification of such fingerprint target and pathway proteins are also described.

Described below are methods for the identification of compounds, which modulate the expression of proteins, involved in the regulation of body weight and/or eating. Additionally described below are methods for the treatment of body weight and/or eating disordered states including, but not limited to, obesity.

Also discussed below are methods for prognostic and diagnostic evaluation of body weight and/or eating disordered states and for the identification of subjects exhibiting a predisposition to such disorders and for identifying the most appropriate therapy for such individuals.

1. Identification of differentially expressed and pathway proteins

In one embodiment, the present invention concerns methods for the identification of proteins which are involved in body weight and/or eating disorders and/or which are involved in obesity. Such proteins may represent

proteins, which are differentially expressed in body weight and/or eating disordered states relative to their expression in normal states. Further, such proteins may represent proteins that are differentially expressed or regulated in response to manipulation relevant to modulating body weight and/or food intake. Such differentially expressed proteins may represent 'target' or 'fingerprint' proteins. Methods for the identification of such proteins are described in section 1.1. Methods for the further characterisation of such differentially expressed proteins and for their identification as target and/or fingerprint proteins are presented below in section 1.3.

In addition, methods are described herein in section 1.3, for the identification of proteins termed pathway proteins involved in body weight and/or eating disordered states and/or obesity. Pathway proteins, as used herein, refer to a protein, which exhibits the ability to interact with other proteins relevant to body weight and/or eating disordered states. A pathway protein may be differentially expressed and therefore may have the characteristics of a target or fingerprint protein.

'Differential expression', as used herein, refers to both qualitative as well as quantitative differences in protein expression. Thus a differentially expressed protein may qualitatively have its expression activated or completely inactivated in normal versus body weight and/or eating disordered state or under control versus experimental conditions. Such a qualitatively regulated protein will exhibit an expression pattern within a given tissue or cell type, which is detectable in either

control or body weight and/or eating disordered subject, but not detectable in both. Alternatively, such a qualitatively regulated protein will exhibit an expression pattern within one or more cell types, which is detectable in either control or experimental subjects but not detectable in both. 'Detectable', as used herein, refers to a protein expression pattern, which is detectable by the technique of differential display 2D electrophoresis, which are well known to those of skill in the art.

Alternatively, a differentially expressed protein may have its expression modulated, i.e. quantitatively increased or decreased, in normal versus body weight or eating disorder states or under control versus experimental conditions. The degree to which expression differs in normal versus body weight and/or eating disordered states or control versus experimental states need only be large enough to be visualised via standard characterisation techniques, such as silver staining of 2D-electrophoretic gels. Other such standard characterisation techniques by which expression differences may be visualised are well known to those skilled in the art. These include successive chromatographic separations of fractions and comparisons of the peaks, capillary electrophoresis and separations using micro-channel networks, including on a microchip.

Chromatographic separations can be carried out by high performance liquid chromatography as described in Pharmacia literature, the chromatogram being obtained in the form of a plot of absorbance of light at 280nm against time of separation. The material giving

incompletely resolved peaks is then re-chromatographed and so on.

5 Capillary electrophoresis is a technique described in many publications, for example in the literature "Total CE Solutions" supplied by Beckman with their P/ACE 5000 system. The technique depends on applying an electric potential across the sample contained in a small capillary tube. The tube has a charged surface, such as 10 negatively charged silicate glass. Oppositely charged ions (in this instance, positive ions) are attracted to the surface and then migrate to the appropriate electrode of the same polarity as the surface (in this instance, the cathode). In this electro-osmotic flow (EOF) of the 15 sample, the positive ions move fastest, followed by uncharged material and negatively charged ions. Thus, proteins are separated essentially according to charge on them.

20 Micro-channel networks function somewhat like capillaries and can be formed by photoablation of a polymeric material. In this technique, a UV laser is used to generate high energy light pulses that are fired in bursts onto polymers having suitable UV absorption 25 characteristics, for example polyethylene terephthalate or polycarbonate. The incident photons break chemical bonds with a confined space, leading to a rise in internal pressure, mini-explosions and ejection of the ablated material, leaving behind voids which form micro- 30 channels. The micro-channel material achieves a separation based on EOF, as for capillary electrophoresis. It is adaptable to micro-chip form, each chip having its own sample injector, separation

column and electrochemical detector: see J.S. Rossier et al, 1999, Electrophoresis 20: pp. 727-731.

Differentially expressed proteins may be further
5 described as target proteins and/or fingerprint proteins.
'Fingerprint proteins', as used herein, refer to a
differentially expressed protein whose expression pattern
may be utilised as part of a prognostic or diagnostic
body weight and/or eating disorder evaluation or which,
10 alternatively, may be used in methods for identifying
compounds useful for the treatment of body weight and/or
eating disordered states. A fingerprint protein may also
have characteristics of a target protein or a pathway
protein.

15
'Target protein', as used herein, refers to a
differentially expressed protein involved in body weight
and/or eating disordered states and/or obesity such that
modulation of the level or activity of the protein may
20 act to prevent the development of the disordered states
including, but not limited to, obesity. A target protein
may also have the characteristics of a fingerprint
protein or a pathway protein.

25 **1.1 Method for the identification of differentially
expressed proteins**

A variety of methods may be used for the identification
of proteins, which are involved in body weight and/or
eating disordered states and/or which may be involved in
30 obesity. Described in Section 1.1.1 are several
experimental paradigms, which may be utilised for the
generation of subjects, and samples, which may be used
for the identification of such proteins. Material from

the paradigm control and experimental subjects may be characterised for the presence of differentially expressed protein sequences as discussed below in Section 1.1.2.

5

1.1.1 Paradigms for the identification of differentially expressed proteins

Among the paradigms that may be utilised for the identification of differentially expressed proteins involved in body weight and/or obesity disordered states are paradigms designed to analyse those proteins that are differentially expressed between normal and body weight and/or eating disordered states including, but not limited to, obesity.

15

In one embodiment of such a paradigm tissue from normal and body weight and/or eating disorder subjects would be compared. Such subjects could include, but would not be limited to, subjects with obesity. It could also involve a comparison of normal subjects and subjects who have resisted the development of obesity despite overfeeding. Appropriate tissues would include, but not be limited to, blood and adipose tissue. It could also include post-mortem samples from subjects. Particularly useful tissues would include brain particularly the hypothalamus, skeletal muscle, liver and adipose tissue.

20

25

Among additional paradigms would include a comparison of obese subjects and obese subjects whose body weight had been reduced by, but not limited to, dietary restriction or dietary modification, anorexic drugs such as sibutramine, Y5 receptor antagonists, leptin and leptin mimetics, MC4 agonists, exercise and thermogenic drugs

30

such as b_3 -adrenoceptor agonists.

Among further paradigms would include a comparison of adipose tissue from various fat depots within the same individuals but typically to be a comparison of subcutaneous and omental adipose tissue since omental adipose tissue is associated with the metabolic and cardiovascular complications of obesity, whereas excess subcutaneous adipose tissue does not carry a similar high risk.

In a further paradigm, which may be utilised for the identification of differentially expressed proteins involved in body weight and/or eating disorders are paradigms designed to analyse those proteins which may be involved in genetic models of obesity. Accordingly, such paradigms are referred to as 'genetic obesity paradigms'.

In the case of mice, for example, such paradigms may identify the proteins regulated either directly or indirectly by the ob/ob, db/db, tub or fat gene products. In rats such a paradigm may identify proteins regulated either directly or indirectly by the fa gene product.

In one embodiment of such a paradigm, test subjects may include ob/ob, db/db, tub/tub or fat/fat experimental mice and lean littermate controls. Test subjects could also include fa/fa and male and female ZDF rats. Samples of tissues such as skeletal muscle, whole brain, hypothalamus, adipose tissue and liver would be obtained. The examples below demonstrate the use of such genetic paradigms in identifying proteins which are differentially expressed in obese animals versus normal animals.

In additional embodiments, ob/ob, db/db, tub/tub and/or fat/fat mice and/or fa/fa and ZDF rats and lean control animals and inbred or outbred strains of rodents may be treated with drugs that reduce body weight, particularly body fat mass. Such drugs include, but are not limited to, appetite suppressants such as sibutramine, fenfluramine, NPY antagonists, melanocortin-4 receptor agonists, orexin antagonists, MCH antagonists, thermogenic drugs such as β_3 -adrenoceptor agonists and anti-obesity agents such as leptin, leptin mimetics and other cytokines such as axokine and brain derived neurotrophic factor. Such a paradigm allows the identification of target proteins.

In a further additional embodiment, ob/ob, db/db, tub/tub and/or fat/fat mice and/or fa/fa and/or ZDF rats and lean controls or inbred or outbred strains of rodents may be offered dietary treatments to either worsen the obese state or reduce the level of obesity or to identify animals that resist dietary obesity. For example, either lean or obese animals could be provided with a high fat diet to exacerbate the obese state.

In one embodiment of such a paradigm, Sprague Dawley rats would be fed on a high fat diet or a cafeteria diet consisting of human snack foods. Some of the rats become obese whilst others resist obesity. Thus, this paradigm can be used to select proteins that are associated with a predisposition to the development of obesity and to proteins associated with an ability to resist dietary obesity. This paradigm can be further refined by incorporating drug treatment or exercise paradigms.

In another embodiment of this paradigm, obese rodents that had been weight reduced by dietary restriction would be allowed access to ad-libitum food whilst a control group of obese rodents would remain dietary restricted.

5 Such a paradigm would allow the identification of proteins associated with the regain of obesity following withdrawal of dietary restriction.

10 Some native animal strains do not exhibit obesity in the wild but do when fed a laboratory chow or other laboratory diets. These include the desert rodents, the spiny mouse and the sand rat. Comparison of animals fed on the natural diet and those fed on a laboratory diet allows identification of proteins associated with body
15 weight disorders and obesity.

Selective breeding can also be used to obtain genetically closely related animals that exhibit different degrees of adiposity and/or metabolic efficiency. Comparisons
20 between these closely related animals allows the identification of proteins associated with body weight disorders and obesity.

A particularly important tissue with respect to the
25 control of feeding behaviour and the regulation of energy balance is the hypothalamus. It is clear that many substances act in this area of the brain to regulate feeding and/or energy balance. These include catecholamines, serotonergic agents, leptin, orexins,
30 melanocyte concentrating hormone, melanocortin receptor agonists and antagonists. It is also clear that there are complex interactions between these various substances.

However, no systematic evaluation of the changes in protein composition of the hypothalamus and its constituent nuclei, which underlay the changes in feeding behaviour, has yet been undertaken. Thus, in a further embodiment, hypothalamic nuclei from fed v fasted rodents; sated v non-sated rodents; obese v lean rodents; fat preferring v non-fat preferring rodents may be used to identify the hypothalamic protein patterns associated with particular feeding behaviours.

10

In addition to whole animal studies, paradigms include systems in which isolated cells such as adipocytes, are incubated in vitro with agents such as leptin.

15

1.1.2 Analysis of paradigm material

In order to identify differentially expressed proteins, tissues from subjects utilised in paradigms such as those described above in 1.1.1 are obtained. In addition, blood and body fluids may be analysed since the differentially expressed proteins might be released into the circulations.

20

Whole tissue or isolated cells may be used. Sub-cellular fractions of cells might also be used. Particularly useful sub-cellular fractions include the nuclear protein fraction.

25

1.2 Methods for the identification of pathway proteins

Methods are described herein for the identification of pathway proteins. 'Pathway protein', as used herein, refers to a protein which exhibits the ability to interact with differentially expressed proteins involved in body weight and/or eating disorders and/or to interact

30

with differentially expressed proteins which are relevant to obesity. A pathway protein may be differentially expressed and, therefore, may have the characteristics of a target and/or fingerprint protein.

5

Any method suitable for detecting protein-protein interactions may be employed for identifying pathway proteins by identifying interactions between proteins and proteins known to be differentially expressed in body weight and/or eating disordered states and/or obesity regulation. Such differentially expressed proteins may be cellular or extracellular proteins. Those proteins, which interact with such differentially expressed proteins, represent pathway gene products.

15

Among the traditional methods, which may be employed, are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Utilising procedures such as these allows for the identification of pathway proteins. Once identified, a pathway protein may be used, in conjunction with standard techniques, to identify its corresponding pathway gene. For example, at least a portion of the amino acid sequence of the pathway gene product may be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, e.g. Creighton (1983)

20

25

'Proteins: Structures and Molecular Principles', W.H. Freeman & Co., N.Y., pp. 34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for pathway gene sequences. Screening may be accomplished, for example, by standard hybridisation or PCR techniques. Techniques for the generation of oligonucleotide mixtures

30

and the screening are well-known (see, e.g. Ausubel, supra. and PCR Protocols: A Guide to Methods and Applications (1990) Innis, M. et al., eds. Academic Press Inc., New York).

5

One method, which detects protein interactions in vivo, the yeast two-hybrid system, is described in detail for illustration only and not by way of limitation. One version of this system has been described (Chien et al
10 (1991) Proc. Natl. Acad. Sci. USA, 88, 9578-9582) and is commercially available from Clontech (Palo Alto, Calif.).

Briefly, utilising such a system, plasmids are constructed that encode two hybrid proteins: one
15 consists of the DNA-binding domain of a transcription activator protein fused to a known protein, in this case, the differentially expressed protein known to be involved in body weight and/or eating disordered states and/or obesity regulation, and the other consists of the
20 transcription activator protein's activation domain fused to an unknown protein that is encoded by a cDNA which has been recombined into this plasmid as part of a cDNA library. The plasmids are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a
25 reporter gene (e.g. lacZ) whose regulatory region contains the transcription activator's binding sites. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function
30 and the activation domain hybrid cannot because it cannot localise to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the

reporter gene, which is detected by an assay for the reporter gene product.

5 The yeast two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with a known differentially expressed 'bait' protein. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of the bait
10 protein product fused to the DNA-binding domain are co-transformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, and not by way of limitation, the bait gene can be cloned into a vector
15 such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins
20 encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait proteins are to be detected can be made using methods routinely practised in the art.
25 According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the activation domain of GAL4. This library can be co-transformed along with the bait-gene GAL4 fusion plasmid
30 into a yeast strain, which contains a lacZ gene driven by a promoter which contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 activation domain, that interacts with bait gene product will reconstitute

an active GAL4 protein and thereby drive expression of the lacZ gene. Colonies which express lacZ can be detected by their blue color in the presence of X-gal. The cDNA can then be purified from these strains, and
5 used to produce and isolate the bait gene-interacting protein using techniques routinely practised in the art.

Once a pathway protein has been identified and isolated, it may be further characterised as, for example,
10 discussed below, in Section 1.3.

1.3 Characterisation of differentially expressed and pathway proteins

Differentially expressed proteins, such as those
15 identified via the methods discussed, above, in Section 1.1, and pathway genes, such as those identified via the methods discussed, above, in Section 1.2, above, as well as genes identified by alternative means, may be further characterised by utilising, for example, methods such as
20 those discussed herein. Such proteins will be referred to herein as 'identified proteins'.

Analyses such as those described herein, yield information regarding the biological function of the
25 identified proteins. An assessment of the biological function of the differentially expressed proteins, in addition, will allow for their designation as target and/or fingerprint proteins.

30 Specifically, any of the differentially expressed proteins whose further characterisation indicates that a modulation of the proteins expressed or a modulation of the proteins activity may ameliorate any of the body

weight and/or eating disorders will be designated 'target proteins', as defined above, in Section 1. Such target proteins, along with those discussed below, will constitute the focus of the compound discovery strategies discussed below, in Section 3. Further, such target proteins and/or modulating compounds can be used as part of the treatment and/or prevention of body weight and/or eating disorders and/or obesity.

Any of the differentially expressed proteins whose further characterisation indicates that such modulations may not positively affect body weight and/or eating disorders, but whose expression pattern contributes to a protein 'fingerprint' pattern correlative of, for example, a body weight and/or eating disordered state, will be designated a 'fingerprint protein'. 'Fingerprint patterns' will be more fully discussed below, in Section 7.1. It should be noted that each of the target proteins may also function as fingerprint proteins, as well as may all or a portion of the pathway proteins.

It should further be noted that the pathway proteins may also be characterised according to techniques such as those described herein. Those pathway proteins which yield information indicating that they are differentially expressed and that modulation of the proteins expression or a modulation of the proteins expression or a modulation of the proteins activity may ameliorate any of the body weight and/or eating disorders of interest will also be designated 'target proteins'. Such target proteins, along with those discussed above, will constitute the focus of the compound discovery strategies discussed below, in Section 3 and can be used as part of

the treatment methods described in Section 4, below.

It should be additionally noted that the characterisation of one or more of the pathway proteins may reveal a lack of differential expression, but evidence that modulation of the gene's activity or expression may, nonetheless, ameliorate body weight and/or eating disorder symptoms. In such cases, these genes and gene products would also be considered a focus of the compound discovery strategies of Section 3 below.

In instances wherein a pathway proteins characterisation indicates that modulation of gene expression or gene product activity may not positively affect body weight and/or eating disorders of interest, but whose expression is differentially expressed and contributes to a gene expression fingerprint pattern correlative of, for example, a body weight and/or eating disordered state, such pathway genes may additionally be designated as fingerprint genes.

A variety of techniques can be utilised to further characterise the identified proteins. First, the corresponding nucleotide sequence of the identified protein may be obtained by utilising standard techniques well known to those of skill in the art, may, for example, be used to reveal homologies to one or more known sequence motifs which may yield information regarding the biological function of the identified protein.

Secondly, the biological function of the identified proteins may be more directly assessed by utilising

relevant in vivo and in vitro systems. In vivo systems may include, but are not limited to, animal systems, which naturally exhibit body weight and/or eating disorder-like symptoms, or ones which have been engineered to exhibit such symptoms. Further, such systems may include systems for the further characterisation of body weight and/or eating disorders, and/or obesity, and may include, but are not limited to, naturally occurring and transgenic animal systems such as those described above, in Section 1.1.1, and Section 2.2.1 below. In vitro systems may include, but are not limited to, cell-based systems comprising cell types known to be associated with energy storage and metabolism. Such cells may be wild type cells, or may be non-wild type cells containing modifications known to, or suspected of, contributing to a body weight and/or eating disorder of interest. Such systems are discussed in detail below, in Section 2.2.2.

In further characterising the biological function of the identified proteins, the expression of these proteins may be modulated within the in vivo and/or in vitro systems, i.e. either overexpressed or underexpressed in, for example, transgenic animals and/or cell lines, and its subsequent effect on the system then assayed. Alternatively, the activity of the identified protein may be modulated by either increasing or decreasing the level of activity in the in vivo and/or in vitro system of interest, and its subsequent effect then assayed.

The information obtained through such characterisations may suggest relevant methods for the treatment of body weight and/or eating disorders involving the protein of

interest. Further, relevant methods for the control of obesity involving the protein of interest may be suggested by information obtained from such characterisations. For example, treatment may include a modulation of protein expression and/or protein activity. Characterisation procedures such as those described herein may indicate where such modulation should involve an increase or a decrease in the expression or activity of the protein of interest. Such methods of treatment are discussed below, in Section 4.

2. Differentially expressed and pathway proteins

Identified proteins, which include, but are not limited to, differentially expressed proteins such as those identified in Section 1.1 above, and pathway proteins, such as those identified in Section 1.2 above, are described herein. Specifically, the amino acid sequences of such identified proteins are described. Further, antibodies directed against the identified protein, and cell- and animal-based models by which the identified proteins may be further characterised and utilised are also discussed in this Section.

2.1 Antibodies specific for differentially expressed or pathway proteins

Described herein are methods for the production of antibodies capable of specifically recognising one or more differentially expressed or pathway protein epitopes. Such antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanised or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a FAb expression library, anti-idiotypic

(anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be utilised as part of body weight and/or eating disorder treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of fingerprint, target, or pathway gene proteins, or for the presence of abnormal forms of such proteins.

For the production of antibodies to a differentially expressed or pathway protein, various host animals may be immunised by injection with a differentially expressed or pathway protein, or a portion thereof. Such host animals may include, but are not limited to, rabbits, mice and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvant such as BCG bacille Calmette-Fuerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunised with an antigen, such as target proteins, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunised by injection with differentially expressed or pathway protein supplemented with adjuvants as also described above.

30

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique, which provides for the production of

antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein (1975, Nature 256; 495-497; and US Pat. No. 4,376,110), the human b-cell
5 hybridoma technique (Kosbor, et al., 1983, Immunology Today 4: 72; Cole, et al., 1983, Proc. Natl. Acad. Sci. USA 80; 2026-2030), and the EBV-hybridoma technique (Cole, et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss Inc., pp. 77-96). Such antibodies
10 may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of
15 production.

In addition, techniques developed for the production of 'chimeric antibodies' (Morrison, et al., 1984, Proc. Natl. Acad. Sci. 81: 6851-6855; Neuberger, et al., 1984,
20 Nature 312: 604-608; Takeda, et al., 1985, Nature 314: 452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is
25 a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

30 Alternatively, techniques described for the production of single chain antibodies (US Pat. No. 4,946,778; Bird, 1988, Science 242: 423-426; Huston, et al., 1988, Proc. Natl. Acad. Sci. USA 85: 5879-5883; and Ward, et al.,

1989, Nature 334: 544-546) can be adapted to produce differentially expressed or pathway protein-single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments, which recognise specific epitopes, may be generated by known techniques. For example, such fragments include, but are not limited to, the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternative, Fab expression libraries may be constructed (Huse, et al., 1989, Science 246: 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

2.2 Cell- and animal-based model systems

Described herein are cell- and animal-based systems, which act as models for body weight and or eating disorders. These systems may be used in a variety of applications. For example, the animal-based model systems can be utilised to identify differentially expressed proteins via one of the paradigms described above, in Section 1.1.1. Cell- and animal-based model systems may be used to further characterise differentially expressed and pathway proteins, as described above in Section 1.3. Such further characterisation may, for example, indicate that a differentially expressed protein is a target protein. Second, such assays may be utilised as part of screening strategies designed to identify compounds which are

capable of ameliorating body weight and/or eating disorder symptoms, as described below. Thus, the animal- and cell-based models may be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in treating such body weight and/or eating disorders. In addition, as described in detail below, in Section 6, such animal models may be used to determine the LD₅₀ and the ED₅₀ in animal subjects, and such data can be used to determine the in vivo efficacy of potential body weight and/or eating disorder treatments, including treatments for obesity.

2.2.1 Animal-based systems

Animal-based model systems of body weight and/or eating disorders may include, but are not limited to, non-recombinant and engineered transgenic animals.

Non-recombinant animal models for body weight and/or eating disorders may include, for example, genetic models. Such genetic body weight and/or eating disorder models may include, for example, mouse models of obesity such as mice homozygous for the autosomal recessive ob, db, fat or tub alleles. It could also include rat models, for example fa/fa rats.

Non-recombinant, non-genetic animal models of body weight and/or eating disorder may include, for example, rats or mice fed on a diet containing a large amount of fat. Such diets could be synthetic diets in which the fat content (by calorific value) is more than 50%. Alternative human foods with a high fat content, such as salami and butter, may be provided to the animals.

Additionally, animal models exhibiting body weight and/or eating disorder-like symptoms may be engineered by utilising, for example, the gene sequences of target proteins such as those described above, in Section 2, in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the animal of interest, or, if endogenous gene sequences of target proteins are present, they may either be overexpressed or, alternatively, may be disrupted in order to underexpress or inactivate gene expression of target proteins.

In order to overexpress the target gene sequence of a target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the animal and cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilised in the absence of undue experimentation.

For underexpression of an endogenous gene sequence of a target protein, such a sequence may be isolated and engineered such that when reintroduced into the genome of the animal of interest, the endogenous gene alleles of the target protein will be inactivated. Preferably, the engineered gene sequence of the target protein is introduced via gene targeting such that the endogenous sequence is disrupted upon integration of the engineered target gene sequence into the animal's genome.

Animals of any species, including, but not limited to,

mice, rats, rabbits, guinea pigs, pigs, mini-pigs, goats and non-human primates, e.g. baboons, squirrel, monkeys, rhesus monkeys and chimpanzees may be used to generate body weight and/or eating disorder animal models.

5

Any technique known in the art may be used to introduce a target gene transgene of a target protein into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, US Pat.No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82: 6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56: 313-321); electroporation of embryos (Lo, 1983, Mol. Cell Biol. 3: 1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57: 717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115: 171-229.

20

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4: 761-763). The transgene may be integrated as a single transgene or in concatamers, e.g. head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236). The regulatory sequences required to such a cell-type specific activation will

30

depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the target gene transgene be
5 integrated into the chromosomal site of the endogenous
target gene, gene targeting is preferred. Briefly, when
such a technique is to be utilised, vectors containing
some nucleotide sequences homologous to the gene of the
endogenous target protein of interest are designed for
10 the purpose of integrating, via homologous recombination
with chromosomal sequences, into and disrupting the
function of, the nucleotide sequence of the endogenous
target gene. The transgene may also be selectively
introduced into a particular cell type, thus inactivating
15 the endogenous gene of interest in only that cell type,
by following, for example, the teaching of Gu et al. (Gu,
H. et al., 1994, Science 265: 103-106). The regulatory
sequences required for such a cell-type specific
inactivation will depend upon the particular cell type of
20 interest, and will be apparent to those of skill in the
art.

Once transgenic animals have been generated, the
expression of the recombinant target gene and protein may
25 be assayed utilising standard techniques. Initial
screening may be accomplished by Southern blot analysis
or PCR techniques to analyse animal tissues to assay
whether integration of the transgene has taken place.
The level of mRNA expression of the transgene in the
30 tissues of the transgenic animals may also be assessed
using techniques which include, but are not limited to,
Northern blot analysis of tissue samples obtained from
the animal, in situ hybridisation analysis, and RT-PCR.

Samples of target protein-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the transgene protein of interest.

5 The target protein transgenic animals that express target gene mRNA or target protein transgene peptide (detected immunocytochemically, using antibodies directed against target protein epitopes) at easily detectable levels should then be further evaluated to identify those
10 animals which display characteristic body weight and/or eating disorder-like symptoms. Such symptoms may include, for example, obesity, hyperphagia, hypophagia, leanness, glucose intolerance, hyperinsulinaemia, and/or non-insulin dependent diabetes. Additionally, specific
15 cell types within the transgenic animals may be analysed and assayed for cellular phenotypes characteristic of body weight and/or eating disorders. Further, such cellular phenotypes may include an assessment of a particular cell types fingerprint pattern of expression
20 and its comparison to known fingerprint expression profiles of the particular cell type in animals exhibiting body weight and/or eating disorders. Such transgenic animals serve as suitable model systems for body weight and/or eating disorders.

25 Once target protein transgenic founder animals are produced (i.e. those animals which express target proteins in cells or tissues of interest and which, preferably, exhibit symptoms of body weight and/or eating
30 disorders), they may be bred, inbred, outbred or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to, outbreeding of founder animals with more than

one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound target protein transgenics that transgenically express the target protein of interest at higher levels because of the effects of additive expression of each target gene transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the possible need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the target protein and the development of body weight and/or eating disorder-like symptoms. One such approach is to cross the target protein transgenic founder animals with a wild type strain to produce an F1 generation that exhibits body weight and/or eating disorder-like symptoms, such as hyperphagia, hypophagia, obesity and leanness. The F1 generation may then be inbred in order to develop a homozygous line, if it is found that homozygous target protein transgenic animals are viable.

2.2.2 Cell-based assays

Cells that contain and express target gene sequences which encode target proteins and, further, exhibit cellular phenotypes associated with an obesity disorder, may be utilised to identify compounds that exhibit an ability to ameliorate body weight and/or eating disorder symptoms. Cellular phenotypes, which may indicate an ability to ameliorate body weight and/or eating disorders, may include, for example, resistance to

insulin.

Further, cell lines which may be used for such assays may also include recombinant, transgenic cell lines. For
5 example, the body weight and/or eating disorder animal models of the invention discussed above, in Section 2.2.1, may be used to generate cell lines, containing one or more cell types involved in body weight and/or eating disorders, that can be used as cell culture models for
10 this disorder. While primary cultures derived from the body weight and/or eating disorder transgenic animals of the invention may be utilised, the generation of continuous cell lines is preferred. For examples of techniques which may be used to derive a continuous cell
15 line from the transgenic animals, see Small, et al., 1985, Mol. Cell Biol. 5: 642-648.

Alternatively, cells of a cell type known to be involved in body weight and/or eating disorders may be transfected
20 with sequences capable of increasing or decreasing the amount of target protein within the cell. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the cell of interest, or, if endogenous gene sequences of the target protein
25 are present, they may either be overexpressed or, alternatively, be disrupted in order to underexpress or inactivate target protein expression.

In order to overexpress a gene sequence of a target
30 protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the cell type of interest. Such regulatory regions will be well known to those of

skill in the art, and may be utilised in the absence of undue experimentation.

5 For underexpression of an endogenous target protein the gene sequence may be isolated and engineered such that when reintroduced into the genome of the cell type of interest, the endogenous target gene alleles will be inactivated. Preferably, the engineered target gene sequence is introduced via gene targeting such that the
10 endogenous target sequence is disrupted upon integration of the engineered target gene sequence into the cell's genome. Gene targeting is discussed above, in Section 2.2.1.

15 Transfection of target protein gene sequence nucleic acid may be accomplished by utilising standard techniques. See, for example, Ausubel, 1989, supra. Transfected cells should be evaluated for the presence of the recombinant target gene sequences, for expression and
20 accumulation of target gene mRNA, and for the presence of recombinant target protein production. In instances wherein a decrease in target protein expression is desired, standard techniques may be used to demonstrate whether a decrease in endogenous target gene expression
25 and/or in target protein production is achieved.

3. Screening assays for compounds that interact with the target proteins

30 The following assays are designed to identify compounds that bind to target proteins, bind to other cellular proteins that interact with a target proteins, and to compounds that interfere with the interaction of the target proteins with other cellular proteins. Such

compounds may include, but are not limited to, other cellular proteins. Methods for the identification of such cellular proteins are described below, in Section 3.2

5

Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, comprising extracellular portions of target protein transmembrane receptors, and members of random peptide libraries (see, e.g. Lam, K.S. et al., 1991, Nature 354: 82-84; Houghten, R. et al., 1991, Nature 354: 84-86) made of D- and/or L-configuration amino acids, phosphopeptides (including, but not limited to, member of random or partially degenerate, directed phosphopeptide libraries: see, e.g., Songyang, Z. et al., 1993, Cell 72: 767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanised, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab'), and FAb expression library fragments, and epitope-binding fragments thereof) and small organic or inorganic molecules.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the target protein, and for ameliorating body weight and/or eating disorders. In instances, for example, whereby a body weight and/or eating disorder situation results from a lower overall level of target protein expression and/or target protein activity in a cell or tissue involved in such a body weight and/or eating disorder, compounds that interact with the target protein may include ones which accentuate or amplify the activity of the bound target protein. Such

compounds would bring about an effective increase in the level of target protein activity, thus ameliorating symptoms. In instances whereby mutations within the target gene cause aberrant target proteins to be made
5 which have a deleterious effect that leads to a body weight and/or eating disorder, compounds that bind target protein may be identified that inhibit the activity of the bound target protein. Assays for testing the effectiveness of compounds, identified by, for example,
10 techniques such as those described in Section 3.1 to 3.3, are discussed below, in Section 3.4.

3.1 In vitro screening assays for compounds that bind to the target proteins

15 In vitro systems may be designed to identify compounds capable of binding the target proteins of the invention. Compounds identified may be useful, for example, in modulating the activity of wild type and/or mutant target proteins, may be useful in elaborating the biological
20 function of the target protein, may be utilised in screens for identifying compounds that disrupt normal target protein interactions, or may in themselves disrupt such interactions.

25 The principle of the assays used to identify compounds that bind to the target protein involves preparing a reaction mixture of the target protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus
30 forming a complex which can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring target protein or the

test substance onto a solid phase and detecting target protein/test compounds complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the target protein may be anchored onto a solid surface, and the test compound, which is not anchored, may be labelled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilised as the solid phase. The anchored component may be immobilised by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilised antibody, preferably a monoclonal antibody, specific for the protein to be immobilised may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the non-immobilised component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g. by washing) under conditions such that any complexes formed will remain immobilised on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilised component is pre-labelled, the detection of label immobilised on the surface indicates that complexes were formed. Where the previously non-immobilised component is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface, e.g. using a labelled antibody specific for the previously non-immobilised component (the antibody, in

turn, may be directly labelled or indirectly labelled with a labelled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected, e.g. using an immobilised antibody specific for target protein or the test compound to anchor any complexes formed in solution, and a labelled antibody specific for the other component of the possible complex to detect anchored complexes.

3.2 Assays for cellular proteins that interact with the target protein

Any method suitable for detecting protein-protein interactions may be employed for identifying novel target protein-cellular or extracellular protein interactions. These methods are outlined in Section 1.2, above, for the identification of pathway proteins, and may be utilised herein with respect to the identification of proteins which interact with identified target proteins.

3.3 Assays for compounds that interfere with target protein/cellular macromolecule interaction

The target proteins of the invention may, in vivo, interact with one or more cellular or extracellular macromolecules, such as proteins. Such macromolecules may include, but are not limited to, nucleic acid molecules and those proteins identified via methods such as those described above, in Section 3.2. For purposes of this discussion, such cellular and extracellular macromolecules are referred to herein as 'binding partners'. Compounds that disrupt such interactions may be useful in regulating the activity of the target

protein, especially mutant target proteins. Such compounds may include, but are not limited to, molecules such as antibodies, peptides, and the like, as described, for example, in Section 3.1 above.

5

The basic principle of the assay systems used to identify compounds that interfere with the interaction between the target protein and its cellular or extracellular binding partner or partners involves preparing a reaction mixture containing the target protein, and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of target protein and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the target protein and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound indicates that the compound interferes with the interaction of the target protein and the interactive binding partner. Additionally, complex formation within reaction mixtures contains the test compound and a mutant target protein. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal target proteins.

30

The assay for compounds that interfere with the interaction of the target and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the target protein or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the target protein and the binding partners, e.g. by competition, can be identified by conducting the reaction in the presence of the test substance, i.e. by adding the test substance to the reaction mixture prior to or simultaneously with the target protein and interactive cellular or extracellular binding partner. Alternatively, test compounds that disrupt pre-formed complexes, e.g. compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the target protein or the interactive cellular or extracellular binding partner, is anchored onto a solid surface, while the non-anchored species is labelled, either directly or indirectly. In practice, microtiter plates are conveniently utilised. The anchored species may be immobilised by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by

coating the solid surface with a solution of the target gene product or binding partner and drying.

Alternatively, an immobilised antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilised species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g. by washing) and any complexes formed will remain immobilised on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilised species is pre-labelled, the detection of label immobilised on the surface indicates that complexes were formed. Where the non-immobilised species is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface, e.g. using a labelled antibody specific for the initially non-immobilised species (the antibody, in turn, may be directly labelled or indirectly labelled with a labelled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt pre-formed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected, e.g. using an immobilised antibody specific for one of the binding components to anchor any complexes formed in solution,

and a labelled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt pre-formed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a pre-formed complex of the target protein and the interactive cellular or extracellular binding partner is prepared in which either the target protein or its binding partners is labelled, but the signal generated by the label is quenched due to complex formation (see, e.g. US Pat. No. 4,109,496 by Rubenstein which utilises this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the pre-formed complex will result in the generation of a signal above background. In this way, test substances, which disrupt target protein/cellular or extracellular binding partner interaction, can be identified.

In a particular embodiment, the target protein can be prepared for immobilisation using recombinant DNA techniques described in Section 2.1 above. For example, the target protein gene coding region can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive cellular or extracellular binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practised in the art and described above, in Section 2.1. This antibody can be labelled with the radioactive isotope

¹²⁵I, for example, by methods routinely practised in the art. In a heterogeneous assay, e.g. the GST-target protein gene fusion protein can be anchored to glutathione-agarose beads. The interactive cellular or extracellular binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labelled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the target protein and the interactive cellular or extracellular binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST-target protein gene fusion protein and the interactive cellular or extracellular binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the target protein/binding partner interaction can be detected by adding the labelled antibody and measuring the radioactivity associated with the beads.

30

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the target protein

and/or the interactive cellular or extracellular binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely practised in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in this Section above, and allowed to interact with and bind to its labelled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labelled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the cellular or extracellular binding partner is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesised.

For example, and not by way of limitation, a target protein can be anchored to a solid material as described above, in this Section by making a GST-target protein gene fusion protein and allowing it to bind to glutathione agarose beads. The interactive cellular or extracellular binding partner can be labelled with a

radioactive isotope, such as ^{35}S , and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-target protein gene fusion protein and allowed to bind. After washing away unbound peptides, labelled bound material, representing the cellular or extracellular binding partner binding domain, can be eluted, purified and analysed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

3.4 Assays for amelioration of body weight and/or eating disorder symptoms

Any of the binding compounds, including but not limited to, compounds such as those identified in the foregoing assay systems, may be tested for the ability to prevent or ameliorate body weight and/or eating disorder symptoms, which may include, for example, obesity. Cell-based and animal model-based assays for the identification of compounds exhibiting such an ability to ameliorate body weight and/or eating disorder symptoms are described below.

First, cell-based systems such as those described above, in Section 2.2.2, may be used to identify compounds, which may act to prevent or ameliorate body weight and/or eating disorder symptoms. For example, such cell systems may be exposed to a compound, suspected of exhibiting an ability to ameliorate body weight and/or eating disorder symptoms, at a sufficient concentration and for a time sufficient to elicit a response in the exposed cells. After exposure, the cells are examined to determine

whether one or more of the body weight and/or eating disorder-like cellular phenotypes has been altered to resemble a more normal or more wild type phenotype, or a phenotype more likely to produce a lower incidence or severity of disorder symptoms.

In addition, animals-based body weight and/or eating disorder systems, such as those described above, in Section 2.2.1, may be used to identify compounds capable of ameliorating body weight and/or eating disorder-like symptoms. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions which may be effective in treating such disorders. For example, animal models may be exposed to a compound, suspected of exhibiting an ability to prevent or ameliorate body weight and/or eating disorder symptoms, at a sufficient concentration and for a time sufficient to elicit such a prevention or amelioration of the body weight and/or eating disorder symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with body weight and/or eating disorders such as obesity.

With regard to intervention, any treatments that reverse any aspect of body weight and/or eating disorder-like symptoms should be considered as candidates for human or companion animal body weight and/or eating disorder therapeutic intervention including the treatment of obesity. Dosages of test agents may be determined by deriving dose-response curves, as discussed in Section 6.1 below.

Similarly any treatments that can prevent the development or redevelopment of body weight and/or eating disorders should be considered as candidates for the prevention of human or companion animal body weight and/or eating disorder therapeutic intervention. Such disorders include, but are not limited to, obesity.

Protein expression patterns may be utilised in conjunction with either cell-based or animal-based systems to assess the ability of a compound to ameliorate body weight and/or eating disorder-like symptoms. For example, the expression pattern of one or more fingerprint proteins may form part of a fingerprint profile, which may then be used in such as assessment. Fingerprint profiles are described below, in Section 7.1. Fingerprint profiles may be characterised for known states, either body weight and/or eating disorder or normal states, within the cell- and/or animal-based model systems. Subsequently, these known fingerprint profiles may be compared to ascertain the effect a test compound has to modify such fingerprint profiles, and to cause the profile to more closely resemble that of a more desirable fingerprint. For example, administration of a compound may cause the fingerprint profile of a body weight and/or eating disorder model system to more closely resemble the control system. Administration of a compound may, alternatively, cause the fingerprint profile of a control system to begin to mimic a body weight and/or eating disorder state, which may, for example, be used in further characterising the compound of interest, or may be used in the generation of additional animal models.

4. Compounds and methods for treatment of body weight

and/or eating disorders

Described below are methods and compositions whereby body weight and/or eating disorder symptoms may be ameliorated. It is possible that body weight and/or eating disorders may be brought about, at least in part, by an abnormal level of target protein, or by the presence of a target protein exhibiting an abnormal activity. As such, the reduction in the level and/or activity of such target protein would bring about the amelioration of body weight and/or eating disorder-like symptoms. Techniques for the reduction of target protein gene expression levels or target protein activity levels are discussed in Section 4.1 below.

Alternatively, it is possible that body weight and/or eating disorders may be brought about, at least in part, by the absence or reduction of the level of target protein expression, or a reduction in the level of a target proteins activity. As such, an increase in the level of target protein gene expression and/or the activity of such proteins would bring about the amelioration of body weight and/or eating disorder-like symptoms. Techniques for increasing target protein gene expression levels or target protein activity levels are discussed in Section 4.2 below.

4.1 Compounds that inhibit expression, synthesis or activity of mutant target proteins

As discussed above, target proteins involved in body weight and/or eating disorders may cause such disorders via an increased level of target protein activity. A variety of techniques may be utilised to inhibit the expression, synthesis, or activity of such target genes

and/or proteins.

For example, compounds such as those identified through assays described above, in Section 3, which exhibit inhibitory activity, may be used in accordance with the invention to prevent or ameliorate body weight and/or eating disorder symptoms. As discussed in Section 3 above, such molecules may include, but are not limited to, peptides (such as, for example, peptides representing soluble extracellular portions of target protein transmembrane receptors), phosphopeptides, small organic or inorganic molecules, or antibodies (including, for example, polyclonal, monoclonal, humanised, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')₂, and FAb expression library fragments, and epitope-binding fragments thereof). Techniques for determination of effective doses and administration of such compounds are described below, in Section 6.1. Inhibitory antibody techniques are further described below, in Section 4.1.2.

Further, antisense and ribosome molecules, which inhibit expression of the target protein gene, may also be used in accordance with the invention to inhibit the aberrant target protein gene activity. Such techniques are described below, in Section 4.1.1; triple helix molecules may be utilised in inhibiting the aberrant target protein gene activity.

4.1.1 Inhibitory antisense, ribosome and triple helix approaches

Among the compounds, which may exhibit the ability to prevent or ameliorate body weight and/or eating disorder symptoms are antisense, ribosome and triple helix

molecules. Such molecules may be designed to reduce or inhibit either wild type, or if appropriate, mutant target protein gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridising to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxy-ribonucleotides derived from the translation initiation site, e.g. between the -10 and +10 regions of the target gene nucleotide sequence of interest, are preferred.

Ribosomes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. (For a review, see Rossi, J., 1994, Current Biology 4: 469-471). The mechanism of ribosome action involves sequence specific hybridisation of the ribosome molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribosome molecules must include one or more sequences complementary to the target protein mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see US Pat. No. 5,093,246, which is incorporated by reference herein in its entirety. As such, within the scope of the invention are engineered hammerhead motif ribosome molecules that specifically and efficiently catalyse endonucleolytic cleavage of RNA sequences encoding target proteins.

Specific ribosome cleavage sites within any potential RNA target are initially identified by scanning the molecule

of interest for ribosome cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short TNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target protein gene, containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridise with complementary oligonucleotides, using ribonuclease protection assays.

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC⁺ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementary to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesised in an alternating
5 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

10 Anti-sense RNA and DNA, ribosome and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. They include techniques for chemically
15 synthesising oligodeoxyribonucleotides and oligo-ribonucleotides well known in the art such as, for example, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be
20 incorporated into a wide variety of vectors, which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesise antisense RNA
25 constitutively inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various well-known modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include,
30 but are not limited to, the addition of flanking sequences or ribo- or deoxy-nucleotides to the 5' and/ or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within

the oligodeoxy-ribonucleotide backbone.

4.1.2 Antibodies for the inhibition of target protein

Antibodies that are both specific for target protein and
5 interfere with its activity may be used to inhibit target
protein function. Where desirable, antibodies specific
for mutant target protein, which interferes with the
activity of such mutant target product, may also be used.
Such antibodies may be generated using standard
10 techniques described in Section 2.3, supra, against the
proteins themselves or against peptides corresponding to
portions of the proteins. The antibodies include, but
are not limited to, polyclonal, monoclonal, Fab
fragments, single chain antibodies, chimeric antibodies,
15 etc.

In instances where the target gene protein is
intracellular and whole antibodies are used,
internalising antibodies may be preferred. However,
20 lipofectin or liposomes may be used to deliver the
antibody or a fragment of the Fab region, which binds to
the target protein epitope into cells. Where fragments
of the antibody are used, the smallest inhibitory
fragment, which binds to the target protein's binding
25 domain, is preferred. For example, peptides having an
amino acid sequence corresponding to the domain of the
variable region of the antibody that binds to the target
protein may be used. Such peptides may be synthesised
chemically or produced via recombinant DNA technology
30 using methods well known in the art (e.g. see Creighton,
1983, supra; and Sambrook et al, 1989, supra).

Alternatively, single chain neutralising antibodies,

which bind to intracellular target protein epitopes, may also be administered. Such single chain antibodies may be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell populating by utilising, for example, techniques such as those described in Marasco et al (Marasco, W. et al, 1993, Proc. Natl. Acad. Sci. USA, 90: 7889-7893).

In instances where the target protein is extracellular, or is a transmembrane protein, any of the administration techniques described below, in Section 6, which are appropriate for peptide administration may be utilised to effectively administer inhibitory target protein antibodies to their site of action.

4.2 Methods for restoring target protein activity

Target proteins that cause body weight and/or eating disorders may be underexpressed within body weight and/or eating disorder situations. Alternatively, the activity of target protein may be diminished, leading to the development of body weight and/or eating disorder symptoms. Described in this Section are methods whereby the level of target protein may be increased to levels wherein body weight and/or eating disorder symptoms are prevented or ameliorated. The level of target protein activity may be increased, for example, by either increasing the level of target protein present or by increasing the level of active target protein, which is present.

For example, a target protein, at a level sufficient to ameliorate body weight and/or eating disorder symptoms

may be administered to a patient exhibiting such symptoms. Any of the techniques discussed below, in Section 6, may be utilised for such administration. One of skill in the art will readily know how to determine the concentration of effective, non-toxic doses of the normal target protein, utilising techniques such as those described below, in Section 4.6.1.

Further, patients may be treated by gene replacement therapy. One or more copies of a normal target protein gene or a portion of the gene that directs the production of a normal target protein with target protein gene function, may be inserted into cells, using vectors which include, but are not limited to, adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes. Additionally, techniques such as those described above may be utilised for the introduction of normal target protein gene sequences into human cells.

Cells, preferably autologous cells, containing normal target protein gene sequences may then be introduced or reintroduced into the patient at positions which allow for the prevention or amelioration of body weight and/or eating disorder symptoms. Such cell replacement techniques may be preferred, for example, when the target protein is a secreted, extracellular protein.

Additionally, antibodies may be administered which specifically bind to a target protein and by binding, serve to, either directly or indirectly, activate the target protein function. Such antibodies can include, but are not limited to, polyclonal, monoclonal, Fab

fragments, single chain antibodies, chimeric antibodies and the like. The antibodies may be generated using standard techniques such as those described above, in Section 2.3, and may be generated against the protein themselves or against proteins corresponding to portions of the proteins. The antibodies may be administered, for example, according to the techniques described above, in Section 4.1.2.

10 **5. Pharmaceutical preparations and methods of administration**

The identified compounds, nucleic acid molecules and cells that affect target protein expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to prevent or to treat or to ameliorate body weight and/or eating disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of body weight and/or eating disorder, including obesity, or alternatively, to that amount of a nucleic acid molecule sufficient to express a concentration of protein which results in the amelioration of such symptoms.

25 **5.1 Effective dose**

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining by ED_{50} (the dose therapeutically effective in 50% of the population) and by determining the ED_{50} of any side-effects (toxicity - TD_{50}). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio TD_{50}/ED_{50} . Compounds,

which exhibit large therapeutic indices, are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected
5 tissue in order to minimise potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for
10 use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration
15 utilised.

5.2 Formulations and use

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional
20 manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration
25 by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral and rectal administration.

For oral administration, the pharmaceutical compositions
30 may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pre-gelatinised maize starch, polyvinylpyrrolidone or

hydroxypropyl methyl-cellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium, stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane,

trichlorofluoromethane, dichloro-tetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurised aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules
5 and cartridges of, e.g. gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

10 The compounds may be formulated for parenteral administration by injection, e.g. by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g. in ampoules or in multi-dose containers, with an added preservative. The
15 compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution
20 with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas,
25 e.g. containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation.
30 Such long acting formulations may be administered by implantation, for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with

suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

5

The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as blister pack. The pack or dispenser device may be accompanied by instructions for administration.

10

6. Diagnosis of the nature of body weight and/or eating disorder abnormalities including obesity

A variety of methods may be employed for the diagnosis of the nature of the body weight and/or eating disorders, the predisposition to body weight and/or eating disorders, and for monitoring the efficacy of any body weight and/or eating disorder compounds during, for example, clinical trials and for monitoring patients undergoing clinical evaluation for the treatment of such disorders.

20

The methods include conventional methods such as waist/hip ratios (BMI), weighing and c.t. or dexascans.

25

Obesity may be detected and the efficacy of treatment monitored by methods and parameters identified by such bodies as the World Health Organisation and the International Obesity Task Force.

30

Methods may also, for example, utilise reagents such as the fingerprint protein described in Section 4.1, and

antibodies directed against differentially expressed and pathway proteins, as described above, in Sections 1.3 (peptides) and 2.3 (antibodies). Specifically, such reagents may be used, for example, for: (1) the
5 detection of the presence of target protein mutations, or (2) the detection of either an over- or an under-abundance of target protein relative to the normal state.

The methods described herein may be performed, for
10 example, by utilising pre-packaged diagnostic kits comprising at least one specific finger print protein or anti-fingerprint protein antibody reagent described herein, which may be conveniently used, e.g. in clinical settings, to diagnose patients exhibiting body weight
15 and/or eating abnormalities.

Any cell type or tissue in which the fingerprint protein is expressed may be utilised in the diagnostics described below. Examples of suitable samples types include cell
20 samples, tissue samples, and fluid samples such as blood, urine or plasma.

Among the methods, which can be utilised herein, are methods for monitoring the efficacy of compounds in
25 clinical trials for the treatment of body weight and/or eating disorders. Such compounds can, for example, be compounds such as those described above, in Section 4. Such a method comprises detecting, in a patient sample, a protein, which is differentially expressed in the body
30 weight and/or eating disorder state relative to its expression in a normal state.

During clinical trials, for example, the expression of a

single fingerprint protein, or alternatively, a fingerprint pattern of a cell involved in a body weight and/or eating disorder can be determined in the presence or absence of the compound being tested. The efficacy of the compound can be followed by comparing the expression data obtained to the corresponding known expression patterns in a normal state. Compounds exhibiting efficacy are those which alter the single fingerprint protein expression and/or the fingerprint pattern to more closely resemble that of the normal state.

The detection of the protein differentially expressed in a body weight and/or eating disorder state relative to their expression in a normal state can also be used for monitoring the efficacy of potential body weight and/or eating disorder compounds and compounds for the treatment of obesity during clinical trials. During clinical trials, for example, the level and/or activity of the differentially expressed protein can be determined in relevant cells and/or tissues in the presence or absence of the compound being tested. The efficacy of the compound can be followed by comparing the protein level and/or activity data obtained to the corresponding known levels/activities for the cells and/or tissues in a normal state. Compounds exhibiting efficacy are those which alter the pattern of the cell and/or tissue involved in the body weight and/or eating disorder to more closely resemble that of the normal state.

EXAMPLE 1

Mouse treatment protocol

Non-fasted lean and obese, 8 week old, female C57 B1/6J ob/ob mice were anaesthetised with 50% "Hyponovel" and

50% "Hyponorm" and then killed humanely with carbon dioxide gas. Liver was removed and snap frozen between tongs in liquid nitrogen. The tissues were lyophilised for 48h, crushed in a mortar with liquid nitrogen and the resultant dried powder stored at -80°C

Protein solubilisation

For analytical 2-D-PAGE, 200 micrograms of dried liver was mixed with 60 microlitres of a solution containing urea (8M), CHAPS (4% w/v), Tris (40mM), DTE (65mM), SDS (0.05% w/v) and a trace of bromophenol blue. A weighed portion of the final diluted sample was loaded into a sample cup at the cathodic end of the IPG gels.

First dimension electrophoresis

A non-linear immobilised pH gradient of IGP strips (3.5-10.0 NL IPG 18cm) was used as the first dimension. It offered high resolution, great reproducibility and allowed high protein loads. Based on specifications of the Geneva University Hospital, the non-linear pH gradient strips were prepared by Amersham-Pharmacia Biotechnology AB and are commercially available. The strips were 3mm wide and 180mm long.

Hydration of the IPG strips was performed overnight in a Pharmacia reswelling cassette with 25ml of a solution of urea (8M), CHAPS (2% w/v), DTE (10mM), Resolyte pH 3.5-10 (2% v/v) and a trace of bromophenol blue.

When the rehydration cassette had been thoroughly emptied and opened, the strips were transferred to the Pharmacia strip tray. After placing IPG strips, humid electrode wicks, electrodes and sample cups in position, the strips

and cups were covered with low viscosity paraffin oil. Samples were applied in the cups at the cathodic end of the IPG strips in a slow and continuous manner, without touching the gel.

5

The voltage was linearly increased from 300 to 3500 V during 3 hours, followed by 3 additional hours at 3500 V, whereupon the voltage was increased to 5000 V. A total volt hour product of 100kvh was used in an overnight run.

10

Second dimension of the electrophoresis

After the first dimension run, the IPG strips were equilibrated in order to resolubilise the proteins and to reduce -S-S- bonds. The strips were thus equilibrated within the strip tray with 100ml of a solution containing Tris-HCl (50mM), pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 min. The SH groups were subsequently blocked with 100 ml of a solution containing Tris-HCl (50mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of bromophenol blue for 5 min.

15

20

In the second dimension run, a vertical gradient slab gel with the Laemmli-SDS-discontinuous system was used with some small modifications, which may be summarised as follows:

25

- Gels are not polymerised in the presence of SDS. This seems to prevent the formation of micelles, which contain acrylamide monomer, thus increasing the homogeneity of pore size and reducing the concentration of unpolymerised monomer in the polyacrylamide. The SDS used in the gel running

30

buffer is sufficient to maintain the necessary negative charge on proteins.

- 5 • Piperazine-diacrylyl (PDA) is used as crosslinker.
This is believed to reduce N-terminal protein blockage, gives better protein resolution, and reduces diammine silver staining background.
- 10 • Sodium thiosulphate is used as an additive to reduce background in the silver staining of gels.
- The combination of the IPG strip and agarose avoids the need for a stacking gel.

15 The gel composition and dimensions were as follows:
Dimensions: 160 x 200 x 1.5mm
Resolving gel: Acrylamide/PDA (9-16%
T/2.6% C)
Stacking gel: No stacking
20 Leading buffer: Tris-HCl (0.375M) pH 8.8
Trailing buffer: Tris-glycine-SDS (25mM-
198mM-0.1% w/v) pH 8.3
Additives: Sodium thiosulphate (5mM)
Polymerisation agents: TEMED (0.05%) APS (0.1%)

25

The gels were poured until 0.7cm from the top of the plates and over-layered with sec-butanol for about two hours. After the removal of the overlay and its replacement with water the gels were left overnight.

30

After the equilibration, the IPG gel strips were cut to size. Six mm were removed from the anodic end and 14mm from the cathodic end. The second dimension gels were

over-layered with a solution containing agarose (0.5% w/v) and Tris-glycine-SDS (25mM-198mM-0.1% w/v) pH 8.3 heated at about 70°C and the IPG gel strips were immediately loaded through it.

5

The gel was run at 8-12°C for 5 hours at a constant current of 40mA/gel. The voltage is non-limiting, but usually requires 100 to 400 V.

10 Staining

Silver staining, which is 100-fold more sensitive than Coomassie Brilliant Blue staining, was used (except where otherwise stated). Thus, the 2-DGE gels were stained with an ammoniacal silver staining as follows:

15

All steps were performed on an orbital shaker at 36 rpm.

Step 1: At the end of the second dimension run, the gels were removed from the glass plates and washed in deionised water for 5 min.

20

Step 2: The gels were soaked in ethanol:acetic acid:water (40:10:50 volume ratio) for 1 hour.

Step 3: The gels were soaked in ethanol:acetic acid:water (5:5:90 volume ratio) for 2 hours or overnight.

25

Step 4: They were washed in deionised water for 5 min.

Step 5: They were soaked in a solution containing glutaraldehyde (1% w/v and sodium acetate (0.5M) for 30 min.

30

Step 6: They were washed 3 times in deionised water for 10 min.

Step 7: In order to obtain homogenous dark brown staining of proteins, gels were soaked twice in a 2, 7-naphthalenedisulphonic acid solution (0.05% w/v) for 30

min.

Step 8: The gels were then rinsed 4 times in deionised water for 15 min.

Step 9: The gels were stained in a freshly made ammoniacal silver nitrate solution for 30 minutes. To
5 prepare 750ml of this solution, 6g of silver nitrate were dissolved in 30ml of deionised water, which was slowly mixed into a solution containing 160ml of water, 10ml of concentrated ammonia (25%) and 1.5ml of sodium hydroxide
10 (10N). A transient brown precipitate might form. After it cleared, water was added to give the final volume.

Step 10: After staining, the gels were washed 4 times in deionised water for 4 min.

Step 11: The images were developed in a solution of
15 citric acid (0.01% w/v) and formaldehyde (0.1% v/v) for 5 to 10 min.

Step 12: When a slight background stain appeared, development was stopped with a solution of Tris (5% w/v) and acetic acid (2% v/v).

20

Scanning of the gels

The Laser Densitometer (4000 x 5000 pixels; 12 bits/pixel) from Molecular Dynamics and the GS-700 from Bio-Rad have been used as scanning devices. These
25 scanners were linked to "Sparc" workstations and "Macintosh" computers.

Quantitative image analysis of the gels using "Melanie II"

30 Two-dimensional polyacrylamide gels may be digitised and analysed by computer to allow quantitative image analysis and automatic gel comparison. Since the 2-D-PAGE technique was first developed in 1975 several computer

systems have been manufactured, mainly by academic 2-D-PAGE related laboratories. In the present work, "Melanie II", developed at the University Hospital of Geneva was used. It is available for "Unix" workstations, as well as for "Power Macintosh" and IBM-compatible computers.

ob/ob and lean mice spot detection, quantitation and matching, gel image extraction, zooming, warping and printing as well as gel stacking and flipping were carried out with the "MelView" program.

The following DEPs were found which were all underexposed in ob/ob mice relative to expression in lean mice.

	LOM 16
15	LOM 17
	LOM 18
	LOM 19
	LOM 20
	LOM 21
20	LOM 22
	LOM 23
	LOM 24
	LOM 25
	LOM 26
25	LOM 27
	LOM 28
	LOM 29

The drawings are presented to show the location of the DEPs by reference to Figure 1 and the differential expression in Figures 2-8.

The locations are shown in the maps of Figures 1A - 1E,

in which Figure 1E shows the entire map from the 2-D-PAGE of liver tissue of lean mice. (It so happens that all the differentially expressed proteins identified are over-expressed in the lean control mice, relative to the ob/ob control mice.) The map is divided into four quadrants in the manner:

Fig. 1A	Fig. 1B
Fig. 1C	Fig. 1D

10 in which the pI runs from left (low pI) to right (high pI) and the relative molecular mass from top to bottom, so that the quadrant of Figure 1A shows the spots corresponding to proteins of the lowest pI and highest relative molecular mass and the quadrant of Figure 1D
15 those of the highest pI and lowest relative molecular mass. The DEPs are marked as LOM16, LOM17, LOM18, LOM19, LOM20, LOM 21, LOM22, LOM23, LOM24, LOM25, LOM26, LOM27, LOM28, and LOM29. The other spots identified are for reference purposes and have numbers of five digits
20 preceded by "P" or "Q", corresponding to the SWISS-PROT accession number, thus linking the spots to their genes. The following Tables 1 and 2 list the characteristics of the DEP spots in terms of relative volumes, areas, and optical densities and apparent pI and relative molecular
25 mass.

Table 1: DEP spots from liver of obese mice

	ID	% Vol.	Area	OD	Pi	RMM
	LOM16	0.177	1.470	1.460	6.35	160851
	LOM17	0.034	0.888	0.595	5.61	90000
5	LOM18	0.059	0.858	0.959	6.54	82896
	LOM19	0.370	3.154	1.414	5.31	58000
	LOM20	0.295	2.603	1.438	5.83	56422
	LOM21	0.409	3.307	1.475	6.31	49972
	LOM22	0.283	2.328	1.492	6.25	42937
10	LOM23	0.245	1.960	1.534	6.80	36508
	LOM24	0.293	2.419	1.462	6.83	32671
	LOM25	0.388	3.277	1.439	6.26	26266
	LOM26	0.298	2.664	1.398	5.10	24202
	LOM27	0.168	2.174	1.012	7.17	15283
15	LOM28	1.495	10.596	1.655	9.07	13016
	LOM29	0.255	2.756	1.200	6.33	11946

Table 2: Reference spots from liver of obese control mice

	ID	% Vol.	Area	OD	Pi	RMM
5	P99015	0.122	1.102	1.286	6.31	160851
	P99015	0.177	1.470	1.460	6.35	160851
	P99015	0.161	0.766	1.076	6.17	160851
	P99015	0.098	1.041	1.252	6.22	160851
	P99015	0.157	1.715	1.302	6.26	160000
10	Q05920	0.085	1.133	1.050	6.16	121447
	Q05920	0.039	0.643	0.803	6.12	121447
	Q05920	0.201	2.266	1.281	6.21	120000
	POSI13	0.023	0.306	0.981	4.87	90604
	POSI13	0.082	0.827	1.214	4.89	89679
15	P08113	0.025	0.337	0.911	4.89	90000
	P99017	0.011	0.766	0.176	5.42	90604
	P99017	0.020	1.194	0.228	5.52	90000
	P99017	0.034	0.088	0.595	5.61	90000
	P99017	0.094	1.317	0.077	5.68	89359
20	P99017	0.020	0.898	0.298	5.77	90000
	P11499	0.033	0.766	0.669	5.02	82305
	P37040	0.020	0.674	0.465	5.28	71490
	P37040	0.008	0.429	0.226	5.25	71299
	P20029	0.030	0.735	0.553	4.99	70356
25	P20029	0.074	0.919	1.072	5.01	70169
	P20029	0.433	3.951	1.391	5.04	69426
	P38647	0.044	1.102	0.539	5.29	69426
	P38647	0.011	0.643	0.200	5.27	69426
	P38647	0.067	1.133	0.861	5.32	69057
30	P38647	0.266	2.909	1.273	5.34	68690
	P16627	0.027	1.164	0.317	5.25	67781
	P16627	0.066	0.949	0.970	5.27	67421
	P16627	0.367	3.675	1.304	5.30	67063
	P07724	0.022	0.888	0.343	5.68	66707

85

P07724	0.133	1.378	1.256	5.79	66529
P07724	0.036	1.072	0.469	5.42	66000

	ID	% Vol.	Area	OD	Pi	RMM
	P07724	0.206	1.929	1.380	5.49	65673
	P14733	0.020	0.766	0.366	5.20	65510
	P14211	0.173	2.113	1.107	4.44	59607
5	P19226	0.050	1.072	0.614	5.26	58289
	P19226	0.370	3.154	1.414	5.31	58000
	P19226	0.132	1.501	1.181	5.29	58144
	P14211	0.023	0.949	0.346	4.43	56578
	P27773	0.077	1.317	0.893	5.70	56422
10	P27773	0.295	2.603	1.438	5.83	56422
	P09103	0.422	3.246	1.471	4.86	55496
	P09103	0.059	0.551	1.332	4.82	55803
	P09103	0.114	1.001	1.421	4.88	55496
	P99019	0.096	1.072	1.252	5.72	54585
15	P02551	0.050	0.827	0.786	5.11	53541
	P04104	0.043	1.041	0.556	5.54	53246
	P20152	0.026	0.521	0.574	5.12	53099
	P56480	0.054	1.072	0.654	4.96	49835
	P56480	0.137	1.409	1.316	5.00	49561
20	P56480	0.448	3.553	1.472	5.05	49152
	P17182	0.468	3.706	1.542	6.34	46771
	P05784	0.038	1.011	0.497	5.15	46771
	P05784	0.083	1.501	0.737	5.20	46771
	P05784	0.130	1.654	1.056	5.26	46642
25	P29758	0.074	1.072	1.017	5.88	44260
	P99021	0.318	2.695	1.392	5.25	42000
	P99022	0.064	0.827	1.093	5.27	42232
	P99016	0.160	1.715	1.218	6.43	41168
	P35505	0.416	3.093	1.635	6.77	40985
30	P14206	0.111	1.562	0.991	4.85	40985
	P99018	0.016	1.164	0.175	5.27	40622
	Q64374	0.484	4.104	1.367	5.08	33929
	P50431	0.058	0.521	1.316	6.78	32060

87

P16015	1.493	9.769	1.722	6.93	28149
P16015	0.560	4.165	1.576	6.84	27855

88

	ID	% Vol.	Area	OD	Pi	RMM
	P16015	0.329	2.909	1.406	6.74	27914
	P00405	0.071	1.409	0.699	4.72	26711
	Q00623	0.020	1.072	0.243	5.45	24664
5	Q00623	0.054	1.592	0.475	5.27	24202
	P14701	0.077	1.746	0.588	4.81	24050
	P02762	0.173	1.776	1.197	4.80	19046
	P02762	0.305	2.542	1.461	4.88	19046
	P99014	0.044	3.553	0.152	4.08	14659
10	P56395	0.236	3.032	1.069	4.83	14567
	P08228	0.468	4.349	1.316	6.14	14537
	P12787	0.224	2.848	1.046	4.91	12433
	P16045	0.173	2.634	0.915	5.06	12381
	P12710	0.255	2.756	1.200	6.33	11946

15

The positions of some of the DEPs coincide with those of reference spots. Sequencing will be undertaken to check whether they are actually the same.

20 Referring now to Figures 2-8, these Figures show images of lean controls (left) and ob/ob ("obese") controls (right) relating to one mouse. It should be appreciated that these images cannot fully represent to the eye the differences in expression measurable by computer. Thus,

25 underneath, a bar chart is provided in which the volume of the spot as a percentage of the total volume of all spots is shown on the y-axis. All bar charts relate to 4 mice and resulted from a student T test ($P < 0.01$).

30

The following proteins have been identified as liver markers.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
5 LOM16	CPS1	Carbamoyl-phosphate synthetase I	P07756/P99015	PMF+MS/MS
LOM17	FTHFD	10-Formyltetrahydrofolate dehydrogenase	P28037/P99017	PMF
LOM18	M2GD	Dimethylglycine dehydrogenase	Q63342	PMF
LOM19	HSP60	Heat shock protein 60	P19226	PMF
LOM20	ERP60	Protein disulfide isomerase ER-60	P27773	PMF
10 LOM21	ALDH1	Aldehyde dehydrogenase, mitochondrial	P47738	PMF
LOM22	SAHH	Adenosylhomocysteinase	P50247	PMF
LOM23	ALR	Alcohol dehydrogenase	P51635	PMF+MS/MS
LOM24	GNMT	Glycine N-methyltransferase	P13255	PMF+MS/MS
LOM25	AOP2	Nonselenium glutathione peroxidase	008709	PMF+MS/MS
15 LOM26	-	Lactoyl glutathione lyase	-	-
LOM27	PPIA	Peptidyl-prolyl cis-trans isomerase A	P17742	PMF+MS/MS
LOM28	FABPL	Fatty acid binding protein, liver	P12710	PMF+MS/MS
LOM29	DDT	D-dopachrome tautomerase	035215	PMF+MS/MS
20 LSEM30	-	Unknown protein	P99032	PMF

Preparative 2-D-PAGE

The analytical 2-DGE described above was repeated, with the following changes. Four mg of dried liver was mixed with 450
25 microlitres of the solubilisation solution and loaded into the IPG strips by in-gel rehydration.

After the first dimension run the strips were equilibrated using 3 ml of each buffer per groove.

30

Protein electroblotting

The blotting of proteins separated by 2-D-PAGE onto

polyvinylidene difluoride (PVDF) membranes has enabled the identification and characterisation of proteins from complex biological samples. Transfer of the proteins can be carried out using several methods such as vacuum, capillary or electric field. Electroblotting, using vertical buffer tanks or a semi-dry method is preferred. Both techniques can use the 3-[cyclohexamino]-1-propanesulfonic acid (CAPS) transfer buffer. Gloves must be worn and all filter papers should be washed three times for 3 min in water and three times in transfer buffer. These two steps are important in order to avoid any protein or amino acid contamination.

The procedure was as follows. After second-dimensional electrophoresis, the gels were soaked in deionized water for 3 min. Then they were equilibrated in a solution containing 10mM CAPS pH 11 for 30 min. At the same time, PVDF membranes were wetted in methanol for 1 min and equilibrated in a solution containing 10mM CAPS pH 11 and methanol (10% v/v) also for 30 min. Electroblotting was carried out in a semi-dry apparatus with a solution containing 10mM CAPS pH 11 and methanol (20% v/v anodic side; 5% v/v cathodic side) at 1 mA/cm² constant current for 3 hours at 15°C.

Protein detection on PVDF membranes

Amido Black and Coomassie Brilliant Blue R-250 were used instead of silver staining to visualise proteins on PVDF membranes and are compatible with the ensuing post-separation analysis. Thus, in another 2-DGE run, after electrotransfer, the PVDF membranes were stained in a solution containing Amido Black (0.5% w/v), isopropanol (25% v/v) and acetic acid (10% v/v) for 2 min. Destaining was done by several soakings in deionized water.

In another run, after electrotransfer, the PVDF membranes were stained in a solution containing Coomassie Brilliant Blue R-250 (0.1% w/v) and methanol (50% v/v) for 15 min. Destaining was done in a solution containing methanol (40%
5 v/v) and acetic acid (10% v/v). The same method was used for preparative gels that did not need electrotransfer for further post-separation analysis, such as peptide mass fingerprinting.

10 The PVDF stained membranes were either air-dried or dried on a 3mm thick plate onto a heating plate at 37°C for 10 min.

Scanning

This was done as described above.

15

Protein identification

In amino sequence analysis by Edman degradation, amino acid derivatives are sequentially cleaved one at a time from the protein. Proteins with a chemically inaccessible alpha-amino
20 group cannot be sequenced directly by this procedure and are termed N-terminally blocked. The best way to overcome the blocked proteins is to generate individual fragments by chemical or proteolytic cleavage. Routinely, ten to twelve Edman degradation cycles were performed for each spot. A
25 search in the SWISS-PROT database was made to detect identity to known protein sequences.

The Amido Black stained proteins were excised with a razor blade and N-terminal sequencing was performed using an ABI
30 model 473A or 477A microsequencer from Applied Biosystems equipped with "'Problott" cartridges.

For internal sequencing, the spots of interest were excised

and soaked for two hours in a solution containing acetic acid (100mM), methanol (10% v/v) and PVP-40 (1% v/v) at 37°C. After three washes in deionized water, the PVDF spots were cut into small pieces (about 1mm²) and incubated in 25 microlitres of a solution containing sodium phosphate (100mM) pH 8.0 and lysyl endopeptidase (1 microgram). Following overnight digestion at room temperature, guanidine-HCl (28 mg) and DTT (100 micrograms) were added. After reduction for 2 hours at 37°C, the mixture was incubated for 30 min, at room temperature, with 300 micrograms of iodoacetamide. The digestion solution was removed and kept. PVDF pieces were then extracted overnight with 25 microlitres of a solution containing isopropanol (70% v/v) and trifluoroacetic acid (5% w/v). This elution solution was removed and the PVDF was washed twice with 60 microlitres of TFA (0.1% w/v). The digestion and elution solutions were pooled together with two final washes and this mixture was separated by two-dimensional reverse phase HPLC and sequence determination performed.

20 Immunoblotting

PVDF membranes were first stained to visualise proteins, following which the immunodetection was undertaken. This allowed matching of proteins detected with ECL against those detected with the non-specific protein stain through computer comparison of both images. The mechanical strength of PVDF was also exploited as the same 2-D gel can be used many times for different antibodies.

The whole procedure was carried out in a rotating oven at room temperature. The use of a nucleic acid glass hybridiser tube minimised the volumes and costs.

- The membranes were blocked in 10 ml of a solution of 30

PBS (pH 7.2) and non-fat dry milk (5% w/v) for 30 min.

- 5 • The membranes were then incubated in 10 ml of a solution containing PBS-"Tween" 20 (0.5% v/v), non-fat dry milk (5% w/v) and the primary antibody/antibodies (1:100 or greater, depending on the antibody) for 2 hours.
- 10 • Three quick rinses were performed with 10 ml of PBS-"Tween" 20 (0.5% v/v) and then the membranes were washed for 3 x 10 min with 10 ml of PBS-"Tween" 20 (0.5% v/v).
- 15 • The membranes were incubated in 10 ml of a solution containing PBS-"Tween" 20 (0.5% v/v), non-fat dry milk (5% w/v) and the secondary peroxidase-conjugated antibody (1:1000; for example, if the primary antibody was sheep anti-mouse, then goat anti-sheep IgG was used as the secondary antibody) for 1 hour.
- 20 • Three quick rinses were performed with 10 ml of PBS-"Tween" 20 (0.5% v/v) and then the membranes were washed for 5 x 10 min with 10 ml of PBS-"Tween" 20 (0.5% v/v).
- 25 • After the last wash, the membranes were transferred to a clean glass plate and covered with 10 ml of developing solution (for example ECL from Amersham International or Roche Diagnostics) for 2 min.
- 30 • The excess developing solution was drained, the 20 membranes were wrapped in "Saran" film and fixed in an X-ray film cassette with the proteins facing up.
- An X-ray film was then exposed in a dark room for few seconds or up to several minutes.

Peptide mass fingerprinting

The 2-DGE method was repeated, but using a Coomassie blue stain. The 2-DGE spots were destained with 100 microlitres of 30% acetonitrile in 50mM ammonium bicarbonate at 37°C for 45 min. The supernatant was discarded and the gel spots dried in a "SpeedVac" for 30 min. The gel spots were rehydrated with 25 microlitres of a solution containing 0.2 micrograms of porcine trypsin and 50mM ammonium bicarbonate for 2 hours at 35°C. Then the gel spots and supernatant were dried in a "SpeedVac" for 30 min, rehydrated with 20 microlitres of H₂O for 30 min at 35°C and dried again for 30 min. Twenty microlitres of a solution of 50% of acetonitrile and 0.1% of TFA was added to the spots and sonicated for 10 min. Two microlitres of the supernatants was loaded in each well of a 96 or 400 MALDI target plate. The samples were air-dried. Then 2 microlitres of a solution containing 4 mg/ml of alpha-cyano-4-hydroxycinnamic acid, 50% acetonitrile and 0.1% TFA was added to each well and air-dried.

The peptide mixtures were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Perseptive Biosystems Voyager Elite MALDI-TOF-MS) with a nitrogen laser (337nm) and operated in reflectron delayed extraction mode.

Protein identification has been carried out using "PeptIdent" (<http://www.expasy.ch/tools/peptident.html>). It is a tool that allows the identification of proteins using pI, relative molecular mass and peptide mass fingerprinting data. Experimentally measured, user-specified peptide masses were compared with the theoretical peptides calculated for all proteins in the SWISS-PROT/TREMBL databases.

MS/MS sequencing

When protein identification was not successful with the peptide mass fingerprinting procedure, the supernatant of digested spots was desalted in "ZipTip" C18 pipette tips (Millipore) and eluted with 50% acetonitrile and 0.1% TFA. Peptides were applied by nanoflow (in-house nanospray) sample introduction to a tandem mass spectrometer that consists of two quadrupoles and an orthogonal time of flight tube (Q-TOF) from MicroMass (UK). Fragment ion spectra were interpreted with the MOWSE database search (<http://www.segnet.dl.ac.uk/mowse.html>).

Data management: The mouse SWISS-2-DPAGE database

SWISS-2-DPAGE is an annotated 2-D-PAGE database in which all the data are easily retrieved by computer programs and stored in a format similar to that of the SWISS-PROT Protein Sequence Database, one of the most updated and annotated protein sequence databases presently available. The SWISS-2-DPAGE database assembles data on proteins identified on various 2-D-PAGE maps. Each SWISS-2-DPAGE entry contains data on one protein, including mapping procedures, physiological and pathological data and bibliographical references, as well as several 2-D-PAGE images showing the protein location. Cross-references are provided to SWISS-PROT and, through the latter, to other databases (EMBL, Genbank, PROSITE, OMIM, etc). The database has been set up on the Expasy World Wide Web server (<http://www.expasy.ch/>). Worldwide, scientists using similar 2-D-PAGE protocols (immobilised pH gradient as first dimensional separation) are now able to compare their images with SWISS-2-DPAGE maps.

EXAMPLE 2

The method of Example 1 was repeated, substituting for the liver tissue 200 micrograms (analytical scale) or 4 mg (preparative scale) of dried gastrocnemius muscle tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. A 2-DGE map was thus obtained, from which DEPs were identified and isolated.

The muscle DEPs were:

10 MOM 31
MOM 32
MOM 34
MOM 36

which were underexpressed in ob/ob mouse skeletal muscle relative to expression in lean mouse skeletal muscle and

15 MOM 33
MOM 35

which were overexpressed in ob/ob skeletal muscle relative to lean. These are shown in Figures 9-11 inclusive.

20

The location of the DEPs has been determined by running the liver extract from lean mice in the same gel as the muscle extract, so that liver protein reference spots appear in the muscle extract gel to serve as reference spots.

25

Table 3 gives the characteristics of the DEP spots in terms of relative volumes, areas, optical densities and apparent pI and relative molecular mass.

30

Table 3

Markers	Vol.	% Vol.	Area	% OD	OD	pI	Mw
MOM31	0.259	0.075	0.919	1.225	1.515	6.21	118405
MOM32	0.339	0.098	1.347	1.087	1.344	6.25	101518
5 MOM33	0.072	0.021	0.643	0.459	0.567	5.19	89359
MOMT35	0.095	0.028	1.011	0.43	0.532	5	40803
MOM34	0.231	0.067	1.133	0.954	1.18	6.19	38941
MOM36	0.091	0.026	1.378	0.301	0.372	6.17	32127

10 The following protein have now been identified as muscle markers.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
15 MOM31	PCX	Pyruvate carboxylase	Q05920	PMF
MOM32	-	2-oxoglutarate dehydrogenase E1 component	-	-
MOM33	-	-	-	-
MOM34	IRP2	Iron responsive element binding protein 2	Q62751	PMF
MOM35	-	-	-	-
20 MOM36	2	-	-	-

EXAMPLE 3

The method of Example 1 was repeated, substituting for the liver tissue 16mg (analytical scale) or 160mg (preparative scale) of white adipose tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. A 2-DGE map was thus obtained, from which DEPs were identified and isolated.

30 The location of the proteins has been determined by running the liver extract from lean mice in the same gel as the white

adipose extract, so that the liver protein reference spots appear in the white adipose extract get to serve as reference spots for the latter also.

5 The following differentially expressed proteins are underexpressed in ob/ob mouse adipose tissue relative to lean mouse adipose tissue:

	WOM 37	WOM 52	WOM 60
	WOM 38	WOM 53	WOM 61
10	WOM 44	WOM 54	WOM 62
	WOM 45	WOM 55	WOM 63
	WOM 48	WOM 57	WOM 64
	WOM 49	WOM 58	
	WOM 50	WOM 59	

15

The following differentially expressed proteins were overexpressed in obese mouse adipose tissue relative to adipose tissue of lean mice:

	WOM 39
20	WOM 40
	WOM 41
	WOM 42
	WOM 43
	WOM 46
25	WOM 47
	WOM 51

These are shown in Figures 12-23 inclusive

30 The characteristics of the DEP spots in terms of relative volumes, areas, and optical densities and apparent pI and relative molecular mass are given in Table 4.

Table 4

	Markers	Vol.	% Vol.	Area	% OD	OD	pI	Mw
5	WOMT37	0.527	0.088	1.164	0.817	2.292	6.11	122912
	WOM38	0.724	0.12	1.654	0.764	2.145	6.97	81427
	WOMT39	0.161	0.027	0.643	0.563	1.581	6.28	68965
	WOM40	0.744	0.124	1.684	0.83	2.328	5.34	67063
	WOMT42	0.277	0.046	0.827	0.576	1.615	5.35	62580
10	WOM41	0.174	0.029	0.735	0.493	1.383	5.29	60937
	WOM43	0.354	0.059	0.766	0.829	2.326	5.12	53967
	WOM46	0.141	0.024	1.011	0.234	0.657	5.15	37256
	WOMT48	0.18	0.03	0.827	0.424	1.191	6.16	36889
	WOM47	0.329	0.055	1.96	0.3	0.841	5.19	36526
15	WOMT49	0.364	0.061	0.949	0.749	2.101	6.37	35371
	WOMT50	9.187	1.529	13.934	1.094	3.069	6.97	32129
	WOM51	0.637	0.106	1.439	0.865	2.426	5.35	31989
	WOM52	1.644	0.274	2.695	1.037	2.909	6.5	26760
	WOM55	0.235	0.039	1.072	0.471	1.322	6.29	22380
20	WSEM65	0.128	0.021	1.746	0.124	0.349	5.39	22172
	WOM54	0.195	0.032	1.409	0.269	0.755	5.66	22275
	WOM53	0.446	0.074	1.47	0.619	1.736	6.01	22069
	WOM56	0.025	0.004	0.613	0.066	0.184	5.53	19279
	WOM57	0.177	0.029	1.133	0.311	0.873	6.5	16454
25	WOM59	0.703	0.117	1.654	0.803	2.252	7.75	14353
	WOM60	1.17	0.195	2.328	0.908	2.548	6.6	13775
	WOM61	0.607	0.101	1.347	0.814	2.283	7.51	13695
	WOM58	0.068	0.011	1.133	0.109	0.305	5.42	13416
	WOM62	0.621	0.103	1.868	0.673	1.887	6.28	11151
	WOM64	0.484	0.081	1.531	0.556	1.561	6.6	10515
	WOM63	0.571	0.095	1.501	0.647	1.816	6.68	10546

The following adipose markers have been identified.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
WOMT37				
WOM38				
WOMT39				
WOM40	GRP75	Mitochondrial stress-70 protein	P38647	PMF + MS/MS
WOM41				
WOMT42	ALB	Albumin (Fragment)	P07724	PMF + MS/MS
WOM43				
WOM44				
WOM45				
WOM46				
WOM47				
WOMT48				
WOMT49				
WOMT50	CA3	Carbonic anhydrase 3 (fragment)	P16015	PMF + MS/MS
WOM51	ANX2	Lipocortin II	P07356	PMF + MS/MS
WOM52	CA3	Carbonic Anhydrase 3 (Fragment)	P16015	PMF
WOM53				
WOM54				
WOM55				
WOM56	FTL1	Ferritin Light Chain 1	P29391	PMF + MS/MS
WOM57				
WOM58				
WOM59				
WOM60				
WOM61	PPcA	Peptidyl-prolyl cis-trans isomerase A	P17742	MS/MS
WOM62	GST P2	Glutathione-S transferase P1 (fragment)	P19157	MS/MS
WOM63				
WOM64	CA2	Carbonic Anhydrase 2 (Fragment)	P00920	PMF + MS/MS
WSEM65				

EXAMPLE 4

The method of Example 1 was repeated, substituting for the liver tissue 400 micrograms (analytical scale) or 4mg (preparative scale) of brown adipose tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. The weight of sample loaded was 150 micrograms (analytical) or 1.5mg (preparative). A 2-DGE map was thus obtained, from which DEPs were identified and isolated.

The location of the proteins has been determined by running the liver extract from lean mice in the same gel as the brown adipose extract, so that the liver protein reference spots appear in the brown adipose extract gel to serve as reference spots for the latter also.

The brown adipose tissue DEPs which are overexpressed in ob/ob mouse brown adipose tissue relative to lean mouse brown adipose tissue are:

20 BOM 66
 BOM 67
 BOM 68
 BOM 75
 BOM 76
25 BOM 77

The brown adipose tissue DEPs that are underexpressed in ob/ob mouse brown adipose tissue relative to lean mouse adipose tissue are:

30 BOM 69
 BOM 70
 BOM 71
 BOM 72

BOM 73

BOM 74

5 The characteristics of the DEP spots in terms of relative volumes, areas, and optical densities and apparent pI and relative molecular mass are given in Table 5.

Table 5

	Markers	Vol.	% Vol	Area	% OD	OD	pI	Mw
10	BOM66	0.592	0.161	2.082	1.178	1.455	6.44	73811
	BOM67	0.043	0.012	0.643	0.329	0.407	5.78	54647
	BOMT68	0.047	0.013	1.194	0.18	0.223	5.68	54476
	BOM69	0.485	0.132	1.623	1.369	1.691	5.13	49744
	BOM71	1.125	0.306	3.277	1.622	2.004	6.64	44857
15	BOM70	0.635	0.173	2.205	1.246	1.539	6.4	44857
	BOM72	0.483	0.131	1.868	1.061	1.311	6.8	38472
	BOM73	0.533	0.145	1.654	1.479	1.827	5.18	24316
	BOM74	0.048	0.013	1.654	0.123	0.152	4.97	12722
	BOM75	0.009	0.002	0.398	0.271	0.335	5.88	12432
20	BOMT76	6.325	1.722	10.627	2.369	2.927	7.61	12093
	BOM77	0.55	0.15	2.787	0.861	1.064	5.14	12111

These are shown in Figures 24-29 inclusive.

25 The following brown adipose tissue markers have been identified:

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
BOM66	ACO2	Aconitate Hydratase (human)	Q99798	MS/MS
BOM67	-	-	-	-
BOMT68	-	-	-	-
BOM69	-	-	-	-
BOM70	-	-	-	-
BOM71	ACO2	Aconitate Hydratase Fragment (human)	A99798	MS/MS
BOM72	FH	Fumarate Hydratase (dimer)	P97807	MS/MS
BOM73	-	-	-	-
BOM74	-	-	-	-
BOM75	-	-	-	-
BOMT76	-	-	-	-
BOMT76	GBP	Lactose-binding Lectin 1	P16045	MS/MS

EXAMPLE 5

Lean mice and ob/ob mice, either fed ad-lib or fasted overnight were anaesthetised with 50% hypnovel and 50% hyponorm and then killed humanely with carbon dioxide gas. The hypothalamus was removed and frozen in liquid nitrogen. The method of example 1 was then followed but using 200mg of hypothalamic tissue rather than 200mg liver.

The references mentioned herein are all expressly incorporated by reference.

Claims:

1. A method of screening an agent to determine its usefulness in treating a condition characterised by abnormal body weight or eating dysfunction, the method comprising:
 - 5 (a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of body weight or eating dysfunction;
 - (b) obtaining a sample of relevant tissue taken from, or
10 representative of, a subject having body weight or eating disorders, who or which has been treated with the agent being screened;
 - (c) determining the presence, absence or degree or expression of the differentially expressed protein or
15 proteins in the tissue from, or representative of, the treated subjects; and
 - (d) selecting or rejecting the agent according to the extent to which it changes the expression of the
20 differentially expressed protein or proteins in the treated subject having body weight or eating disorders.
2. The method of claim 1, wherein the agent is selected if
25 it converts the expression of the differentially expressed protein towards that of a subject having a more normal body weight or eating behaviour.
3. The method of claim 1 or claim 2, wherein the agent is
30 selected if it converts the expression of the protein or proteins to that of the normal subject.
4. The method of any one of claims 1 to 3, wherein the body weight or eating disorder is a result of a disorder which causes an increase in body weight and/or which is associated

with an excess food consumption.

5 5. The method of any one of the preceding claims, wherein the paradigm is based on tissue from obese subjects and normal subjects.

10 6. The method of any one of the preceding claims, wherein the paradigm is based on a comparison of subcutaneous and omental adipose tissue from the same individuals.

15 7. A method of any of claims 1 to 4, wherein the body weight and/or eating disorder is a result of a disorder which causes a reduction in body weight and/or which is associated with a low food intake.

20 8. A method of claim 7, wherein the paradigm is based on tissue from subjects with anorexia nervosa or bulimia or AIDS or cancer and normal subjects.

25 9. The method of claim 1, whereon the paradigm is based on animals which are models of obesity as a result of a genetic mutation such as ob/ob, db/db, agouti, fat, tub, fa/fa together with lean littermates.

30 10. The method of claim 1, wherein the paradigm is based on animals in which obesity is induced or exacerbated by dietary treatment.

35 11. The method of claim 1, wherein the paradigm is based on lean and obese animals obtained by a selective breeding programme from a common stock.

12. The method of claim 1, wherein the paradigm is based on

desert rodents such as spiny mice or sand rats, which develop obesity on normal laboratory diets.

13. The method of claim 1, wherein differential levels of obesity occur in apparently similar animals in which it is attempted to induce obesity by dietary modification.

14. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of body weight or function comprise normal subjects and obese subjects.

15. A method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of body weight comprise obese subjects and body weight reduced previously obese subjects or obese subjects and subjects who resisted the development of obesity.

16. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of body weight comprise normal subjects and subjects having a below normal body weight.

17. The method of claim 15, wherein the body weight of a subject is reduced by treatment with a drug, dietary restriction or exercise.

18. The method of claim 17, wherein the drug is a thermogenic drug.

19. The method of claim 18, wherein the thermogenic drug is a β_3 -adrenoceptor agonist or leptin.

20. The method of claim 17, wherein the drug is an anorexic drug.
21. The method of claim 20, wherein the anorexic drug is
5 sibutramine or fenfluramine or leptin.
22. The method of claim 1 to 4, wherein the paradigm is based on animals, which are fed, fasted or sated.
- 10 23. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise normal subjects and underweight or overweight subjects.
- 15 24. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise:
- (a) normal subjects and underweight or overweight subjects; and
 - 20 (b) underweight or overweight subjects which have not been treated with the agent and underweight or overweight subjects which have been treated with the agent.
- 25 25. The method of claim 24, wherein the differential levels of protein expression are not observed in normal subjects who have and have not been treated with the agent.
- 30 26. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise:
- (a) normal subjects who have and have not been treated with the agent; and
 - (b) subjects having body weight and/or eating disordered

function who have and have not been treated with the agent.

27. The method of claim 26, wherein the differential levels of protein expression are not observed in normal subjects and subjects having body weight and/or eating disordered function, both groups of subject being untreated with the agent.

28. The method of any one of the preceding claims, wherein the paradigm is established using two-dimensional gel electrophoresis carried out on the relevant tissue or a protein-containing extract thereof.

29. The method of any one of the preceding claims, further comprising the step of isolating a differentially expressed protein identified in the method.

30. The method of claim 29, further comprising the step of characterising the isolated protein.

31. The method of any one of the preceding claims, wherein the differentially expressed protein or proteins comprise one or more of MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM 49, WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM 42, WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68, BOM 75, BOM 76, BOM 77, BOM 69, BOM 70, BOM 71, BOM 72, BOM 73, BOM 74.

32. The method of claim 30 or 31, further comprising using the protein in an assay for specific binding partners of the protein.

33. The method of claim 30 or claim 31, further comprising using the protein in an assay to screen for agonists or antagonists of the protein.
- 5 34. The method of any one of claims 1 to 33, wherein the agents or proteins are screened using a high throughput screening method.
- 10 35. A method of making a pharmaceutical composition which comprises having identified an agent using the method of any one of claims 1 to 28, the further step of manufacturing the agent and formulating it with an acceptable carrier to provide the pharmaceutical composition.
- 15 36. A protein for use in a method of medical treatment wherein the protein is selected from MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM 49, WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM 42, WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68, BOM 75, BOM 76, BOM 77, BOM 69, BOM 20 70, BOM 71, BOM 72, BOM 73, BOM 74.
- 25 37. Use of an agent identified by the method of any one of claims 1 to 28 for the preparation of a medicament for the treatment of a condition characterised by body weight and/or eating dysfunction.
- 30 38. The use of claim 37 wherein the condition is obesity, non-insulin dependent diabetes, anorexia nervosa, bulimia or cachexia induced by AIDS or cancer or trauma.
39. The use of claim 37 or claim 38, wherein the

differentially expressed protein or proteins comprise one or more of MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM 49, WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM 42, WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68, BOM 75, BOM 76, BOM 77, BOM 69, BOM 70, BOM 71, BOM 72, BOM 73, BOM 74.

40. A method of treating a condition characterised by body weight and/or eating dysfunction in a patient, the method comprising administering a therapeutically or prophylactically effective amount of such an agent identified by a method of any one of claim 1 to 28 to the patient.

41. The method of claim 40, wherein the body weight and/or eating dysfunction is a result of obesity, non-insulin dependent diabetes or type 2 diabetes, anorexia nervosa, bulimia or cachexia induced by AIDS or cancer or trauma.

42. A method of determining the nature or degree of body weight and/or eating dysfunction in a human or animal subject, the method comprising:

(a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of body weight and/or eating function;

(b) obtaining a sample of the tissue from the subject;

(c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the sample; and

(d) relating the determination to the nature or degree of the body weight or eating dysfunction by reference to a

previous correlation between such a determination and clinical information.

5 43. The method of claim 42, wherein the sample is a tissue sample or body fluid sample or urine.

10 44. The method of claim 42 or claim 43, wherein in the paradigm at least four proteins are differentially expressed, providing a multi-protein fingerprint of the nature or degree of the body weight or eating dysfunction.

15 45. The method of any one of claims 42 to 44, which further comprises determining an effective therapy for treating the body weight or eating dysfunction.

20 46. A method of treatment by the use of an agent that will restore the expression of one or more differentially expressed proteins in the body weight or eating dysfunction state to that found in the normal state in order to prevent the redevelopment of obesity in body weight reduced previously obese subjects.

25 47. A method whereby the pattern of differentially expressed proteins in a tissue sample or body fluid sample or urine of an individual with body weight eating dysfunction is used to predict the most appropriate and effective therapy to alleviate the body weight or eating dysfunction state and to monitor the success of that treatment.

30 48. A method of claim 47, wherein the body weight or eating dysfunction state is obesity.

49. A protein which is differentially expressed in relevant

tissue from, or representative of subjects having differential levels of body weight or eating dysfunction and which is as obtainable by the method of two-dimensional gel electrophoresis carried out on said tissue or a protein-containing extract thereof, the method comprising:

- 5 (a) providing non-linear immobilized pH gradient (IPG) strips of acrylamide polymer 3 mm x 180 mm;
- (b) rehydrating the IPG strips in a cassette containing 25 ml. of an aqueous solution of urea (8M), 3-
10 [(cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of acids and bases of pH 3.5 to 10 (2% w/v) and a trace of Bromophenol Blue;
- (c) emptying the cassette of liquid, transferring the
15 strips to an electrophoretic tray fitted with humid electrode wicks, electrodes and sample cups, covering the strips and cups with low viscosity paraffin oil;
- (d) applying 200 micrograms of an aqueous solution of
20 dried, powdered material of the relevant body tissue in urea (8M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM), SDS (0.05% w/v) and a trace of Bromophenol Blue to the sample cups, at the cathodic end of the IPG strips;
- (e) carrying out isoelectric focusing on the gel at a
25 voltage which increases linearly from 300 to 3500 V during 3 hours, followed by another 3 hours at 3500 V, and thereafter at 5000V for a time effective to enable the proteins to migrate in the strips to their pI-dependent final positions;
- (f) equilibrating the strips within the tray with 100 ml
30 of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 minutes;
- (g) replacing this solution by 100 ml. of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M),

glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of Bromophenol Blue for 5 minutes;

(h) providing a vertical gradient slab gel 160 x 200 x 1.5 mm of acrylamide/piperazine-diacrylyl cross-linker (9-
5 16%T/2.6%C), polymerised in the presence of TEMED (0.5% w/v), ammonium persulphate (0.1% w/v) and sodium thiosulphate (5 mM), in Tris-HCl (0.375M) pH 8.8 as leading buffer;

(i) over-layering the gel with sec-butanol for about 2 hours, removing the overlay and replacing it with water;

10 (j) cutting the IPG gel strips to a size suitable for the second dimensional electrophoresis, removing 6 mm from the anode end and 14 mm from the cathode end;

(k) over-layering the slab gel with an aqueous solution of agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1%
15 w/v) as leading buffer, heated to 70°C and loading the IPG gel strips onto the slab gel through this over-layered solution;

(l) running the second dimensional electrophoresis at a constant current of 40 mA at 8-12°C for 5 hours; and

(m) washing the gel.

20

50. The protein of claim 49, wherein the protein is selected from MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM 49,
25 WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM 42, WOM 43, WOM 46, WOM 47, WOM 51, WOM 66, WOM 67, WOM 68, WOM 75, WOM 76, WOM 77, WOM 69, WOM 70, WOM 71, WOM 72, WOM 73, WOM 74.

30 51. A differentially expressed protein having one or more of the identifying characteristics as set out any one of Tables 3 to 5.

114

52. The protein of claim 51, wherein the identifying characteristics are pI and Mw.

Figure 1A

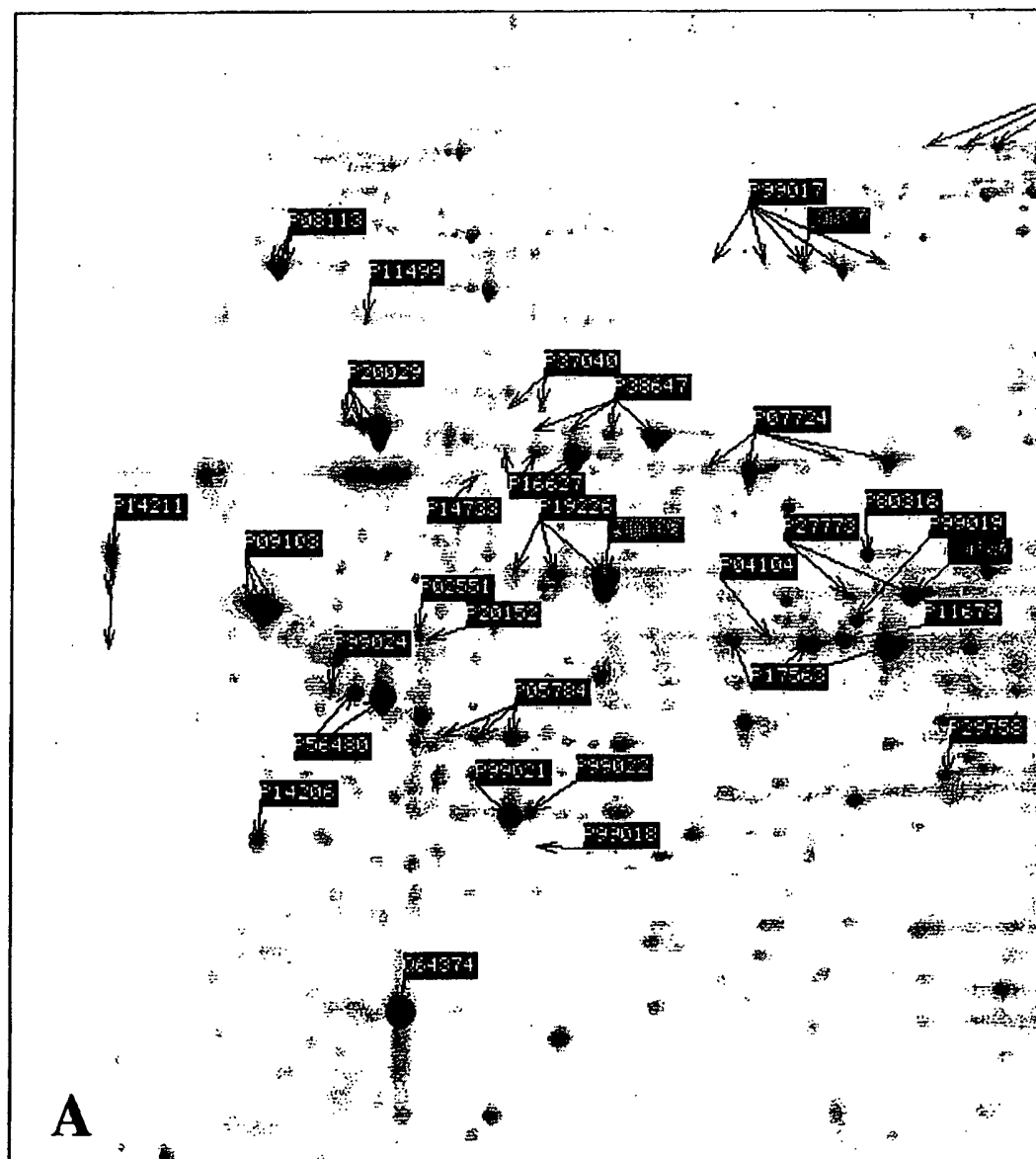


Figure 1 B

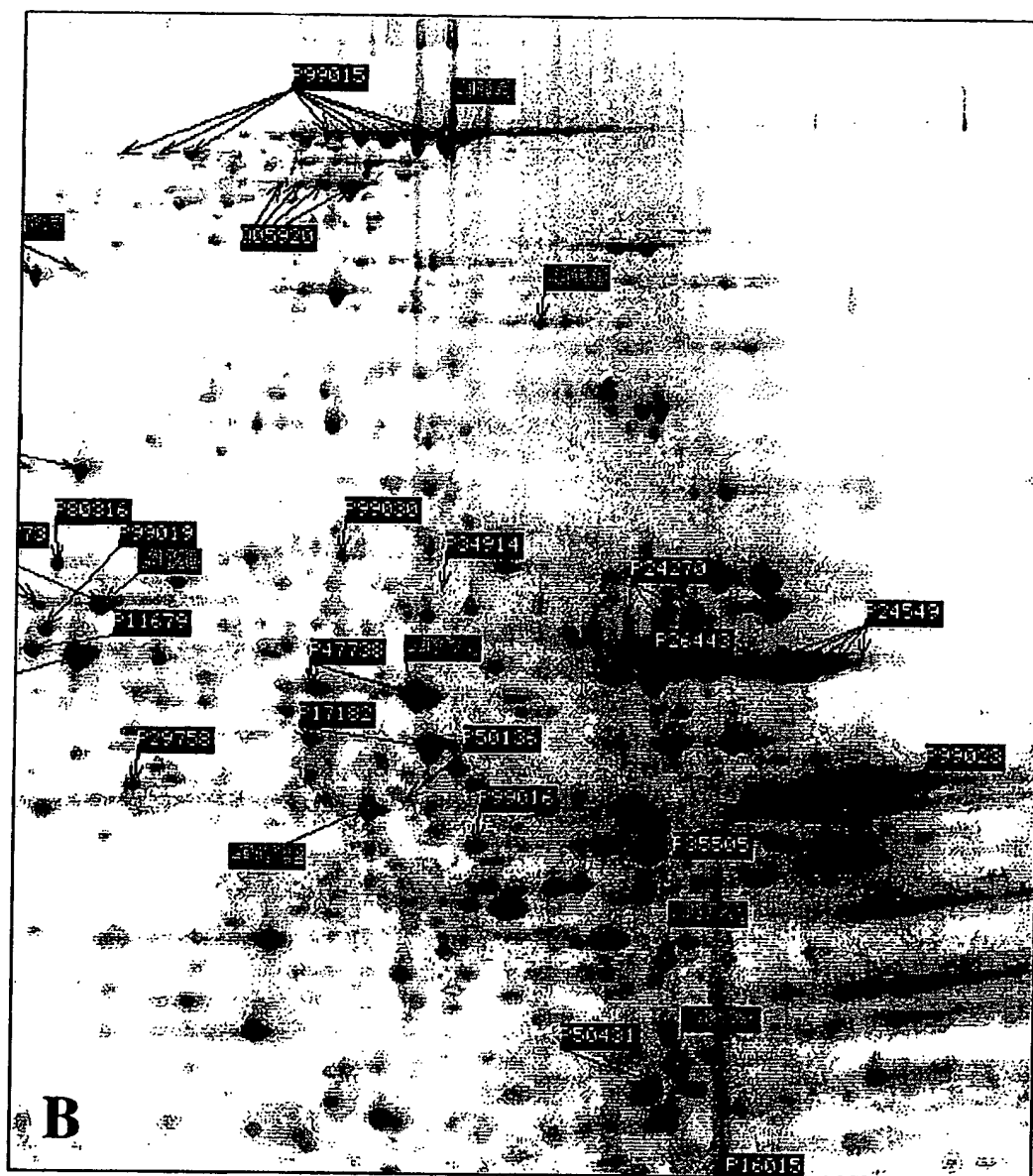


Figure 1c

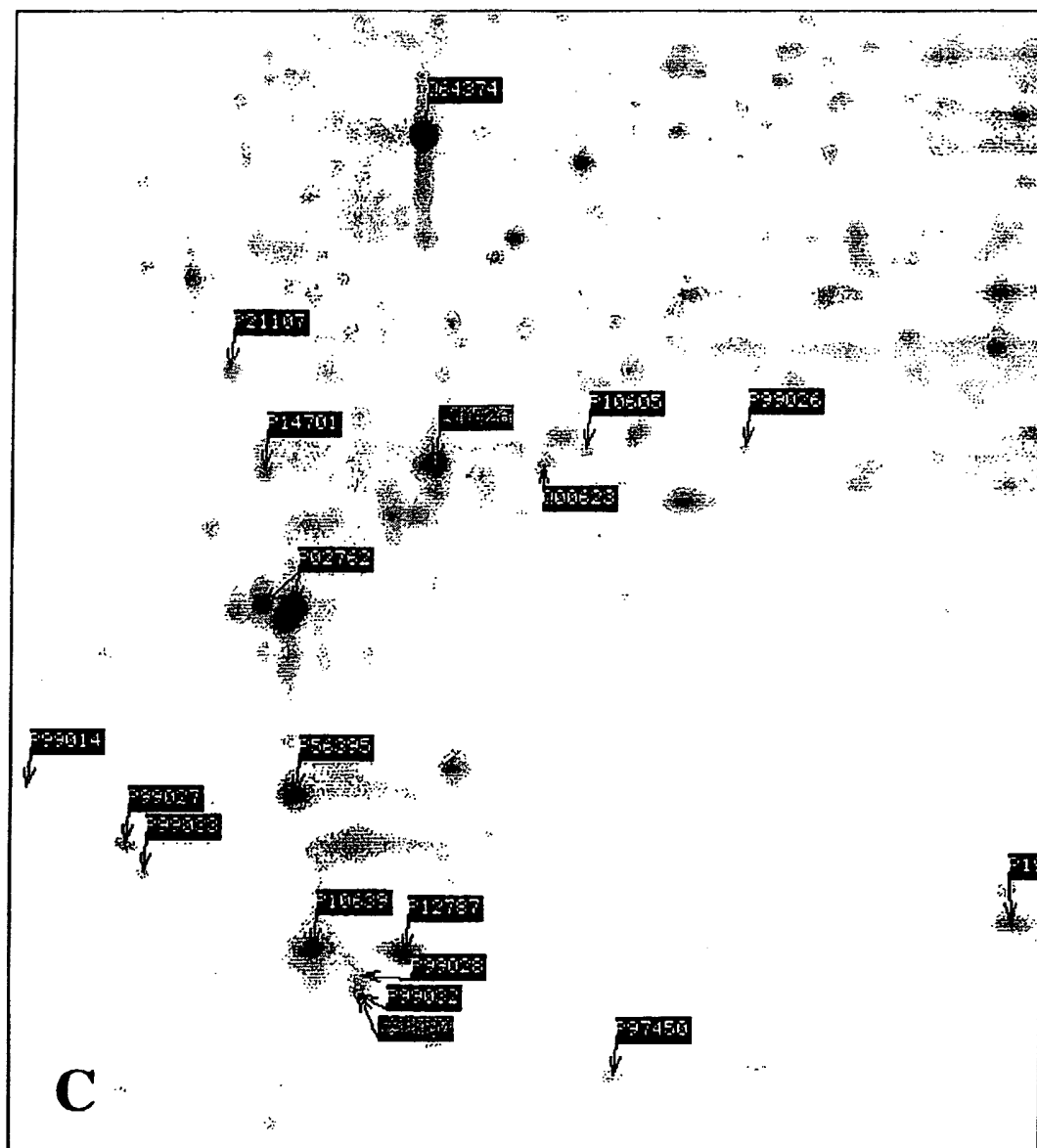


Figure 1D

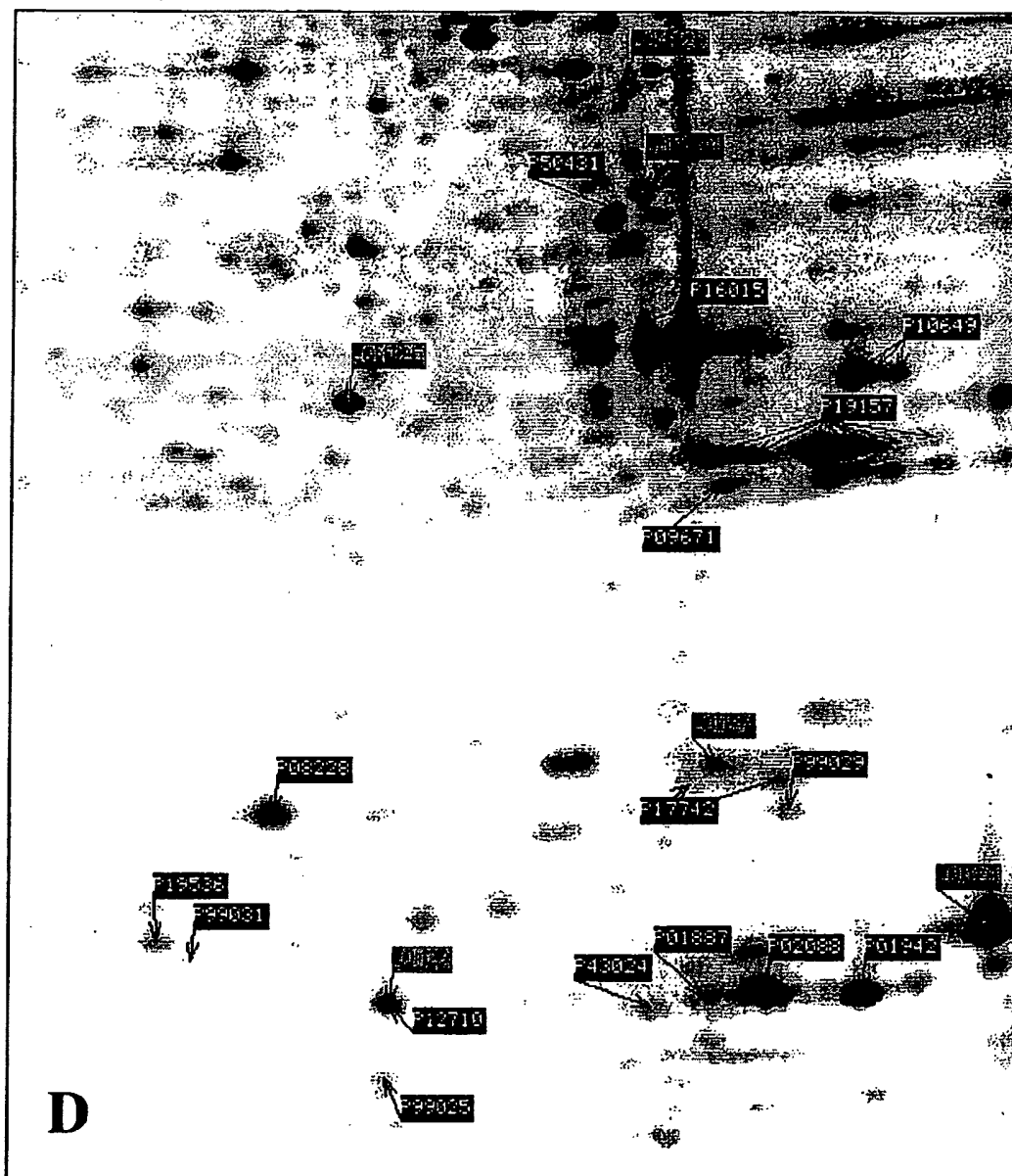


Figure 1E

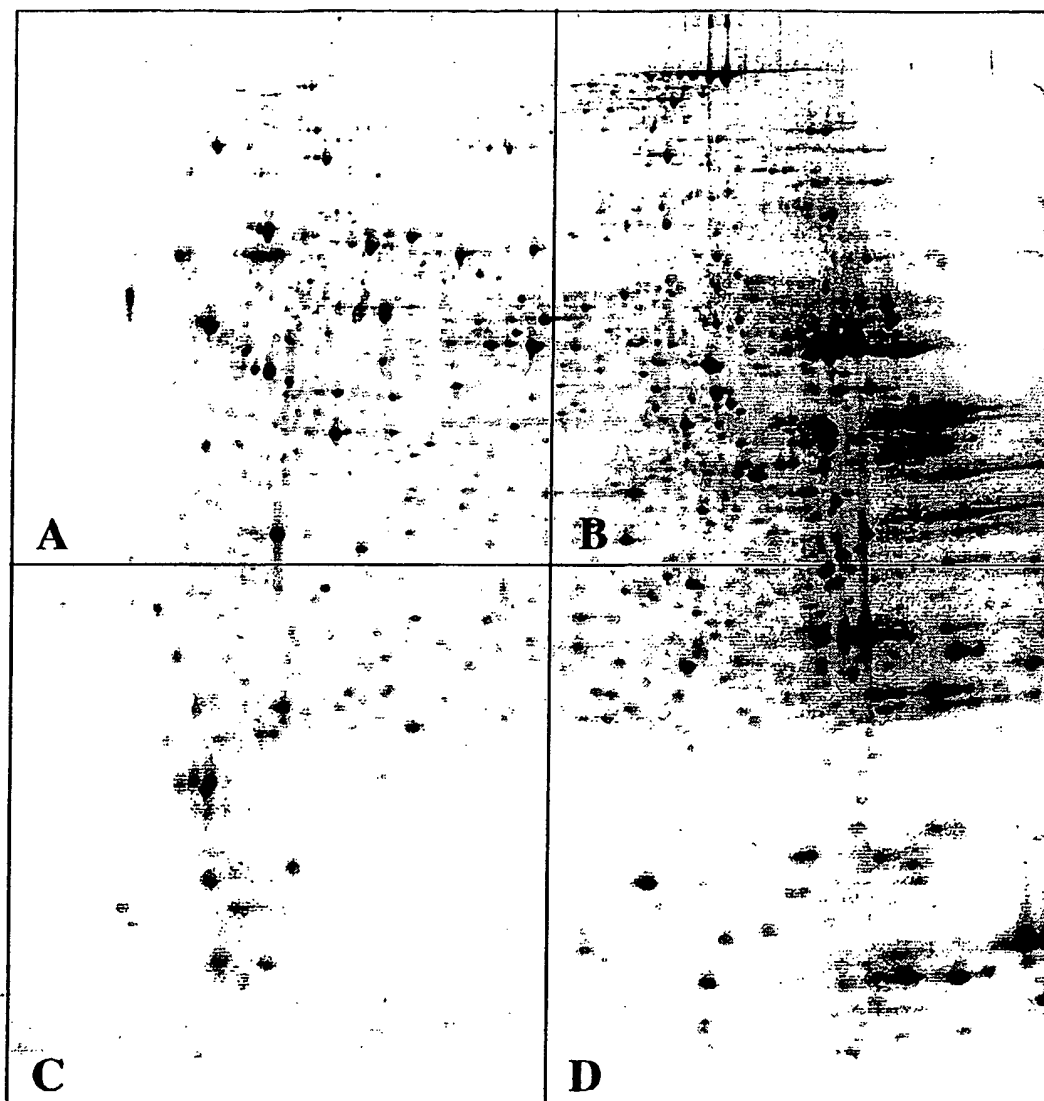


Figure 2

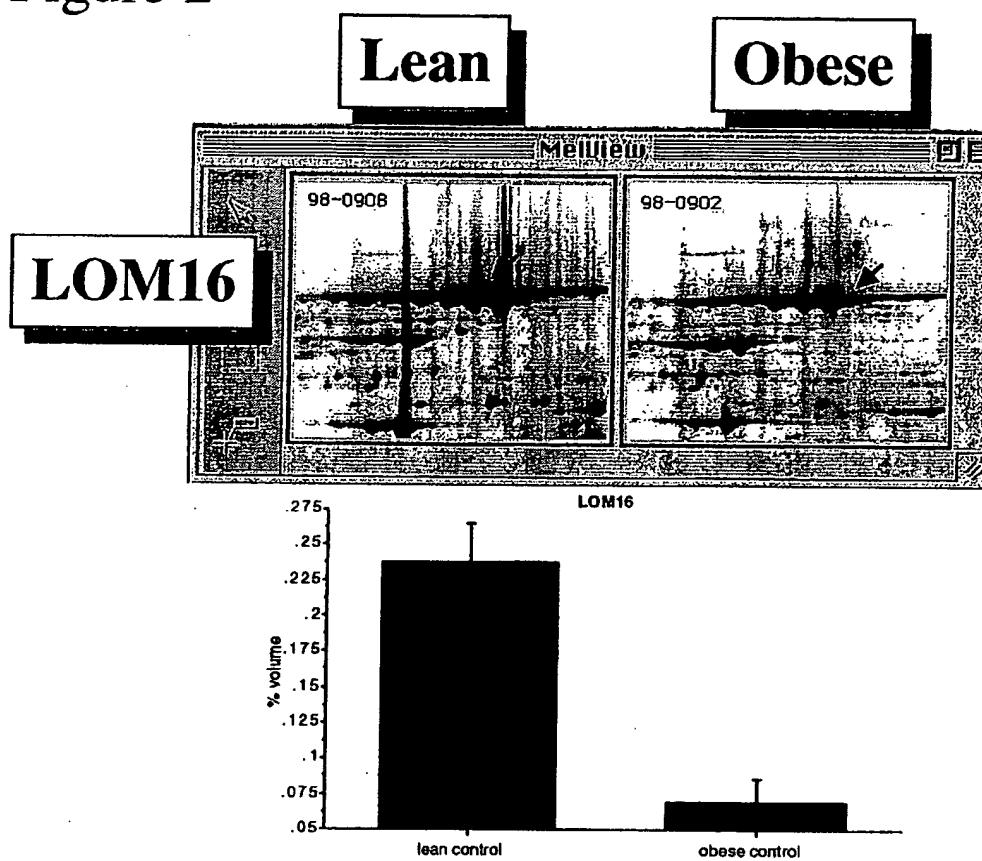


Figure 2 continued

Lean

Obese

LOM17

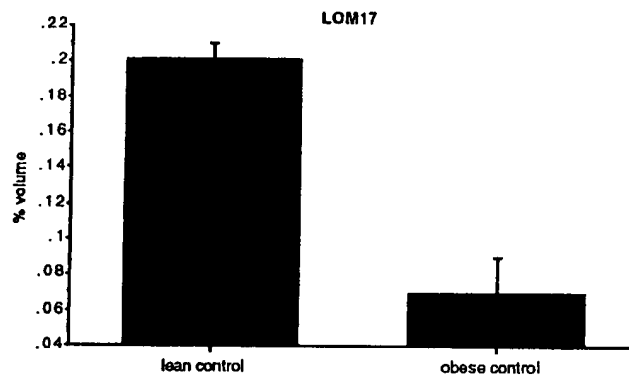
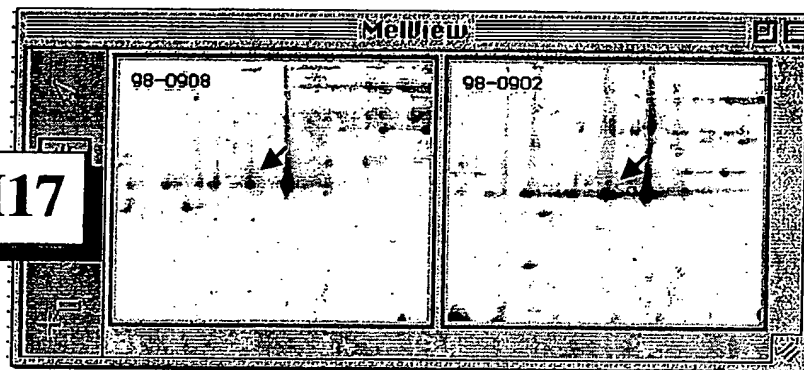


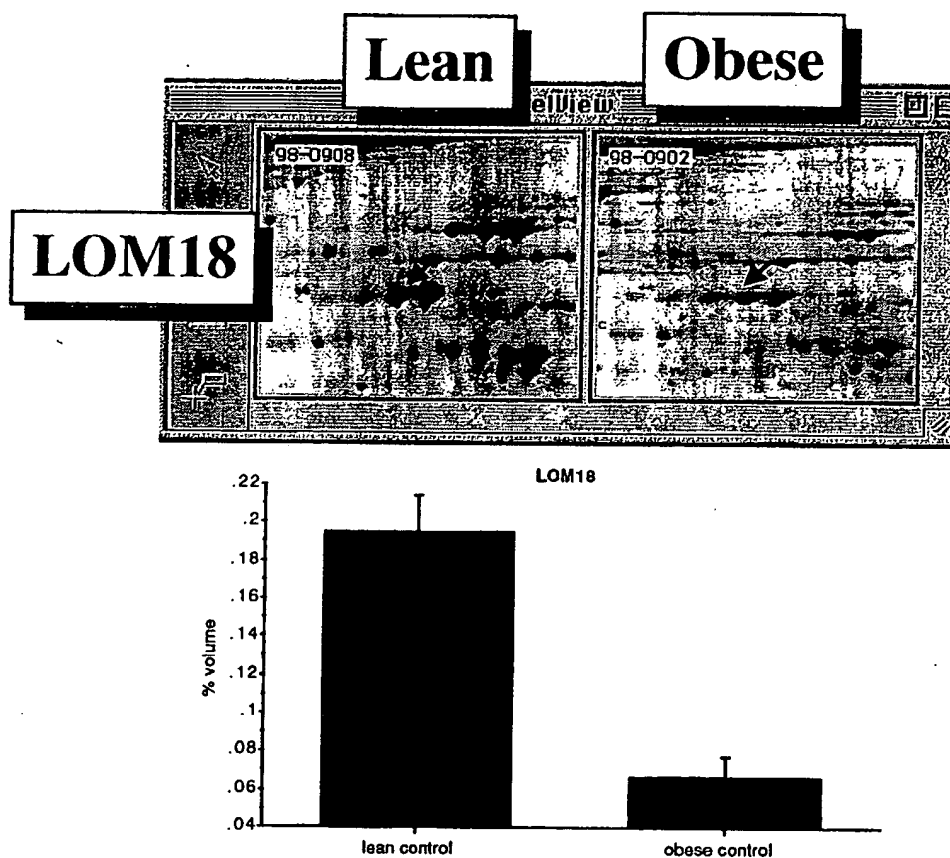
Figure 3

Figure 3 continued

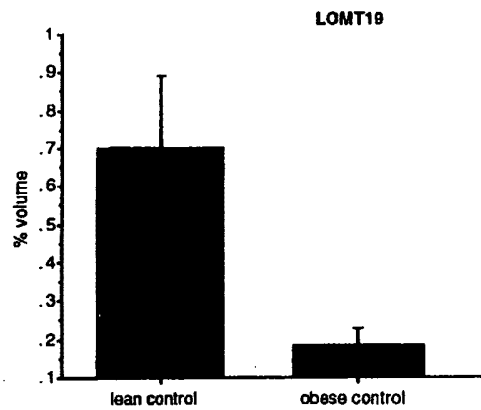
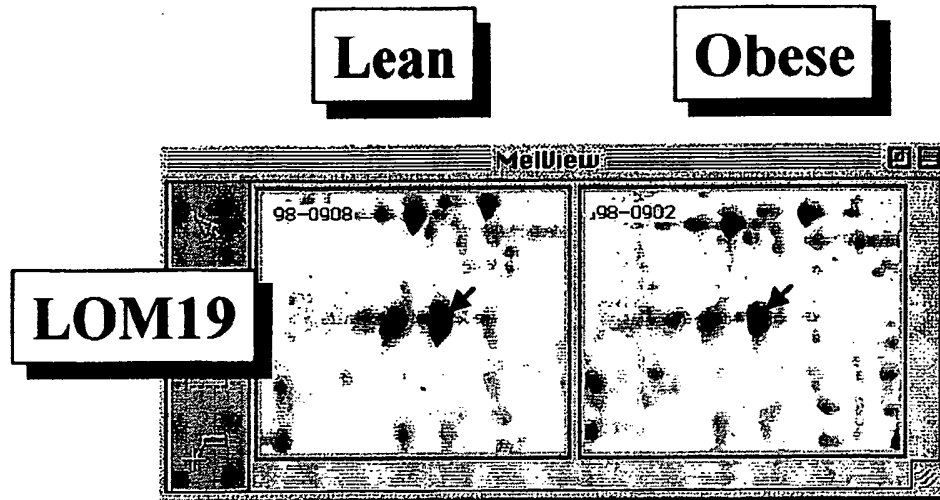


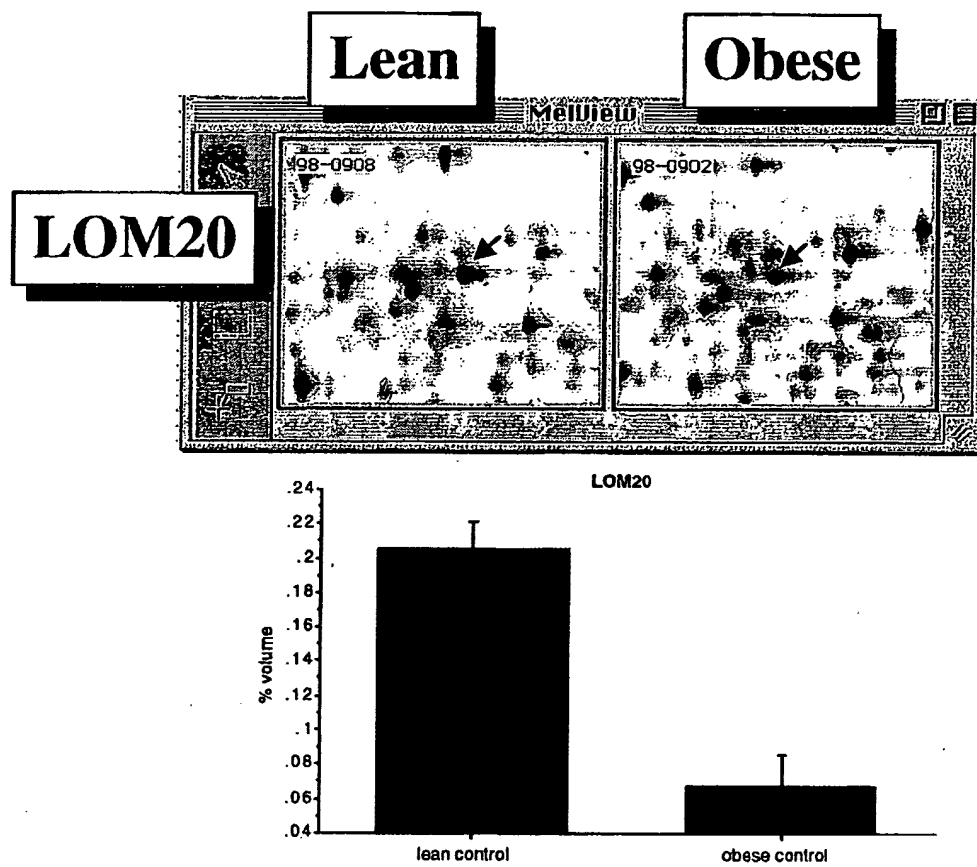
Figure 4

Figure 4 continued

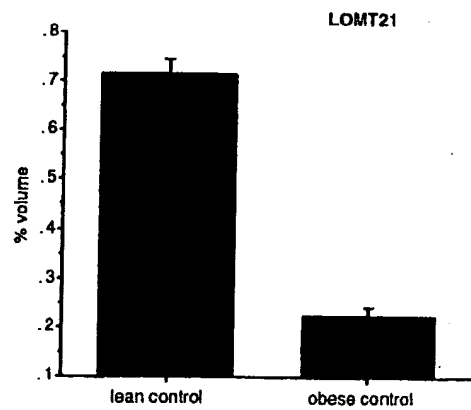
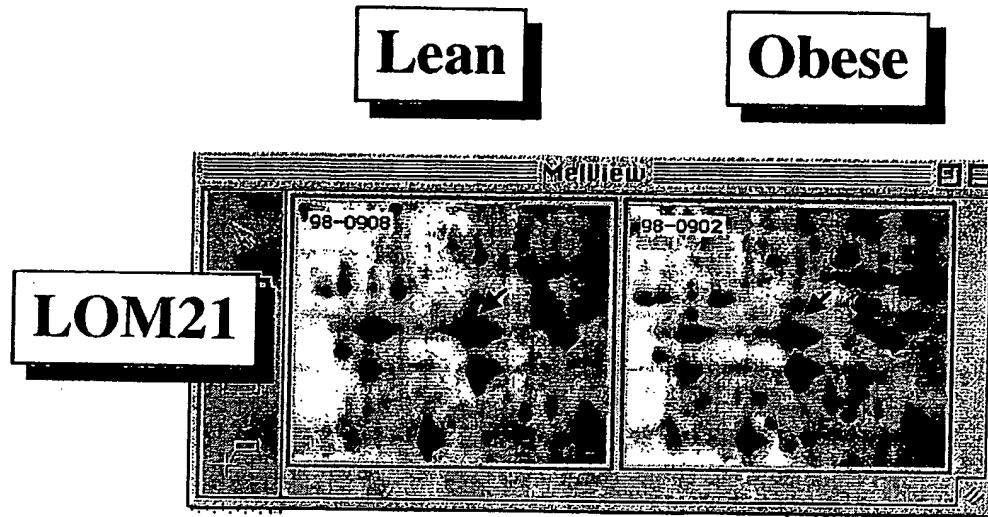


Figure 5

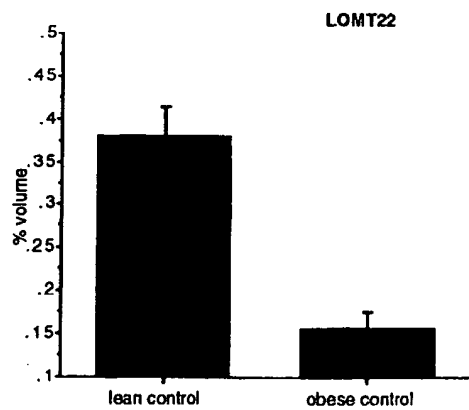
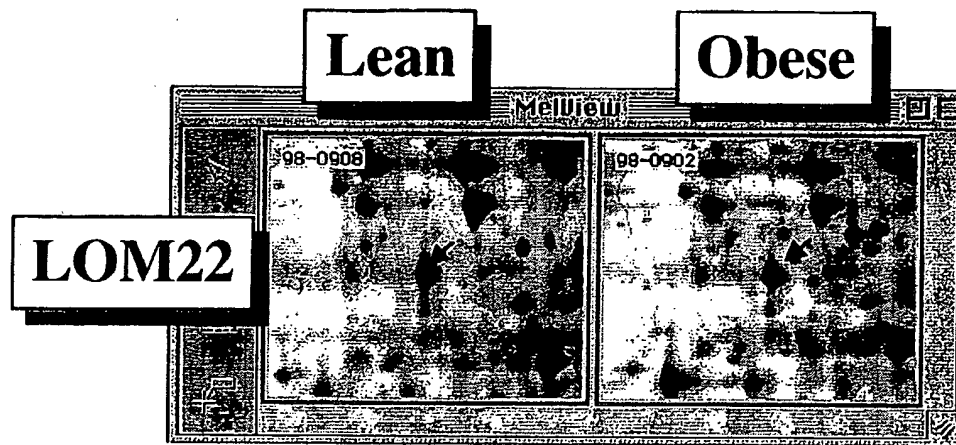


Figure 5 continued

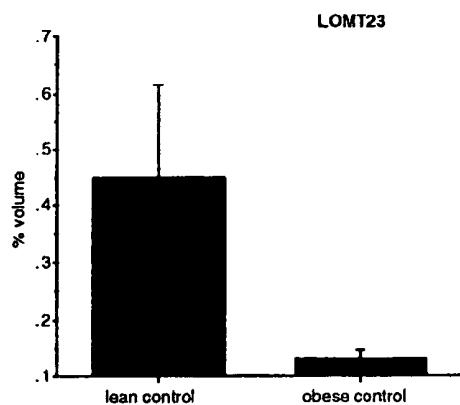
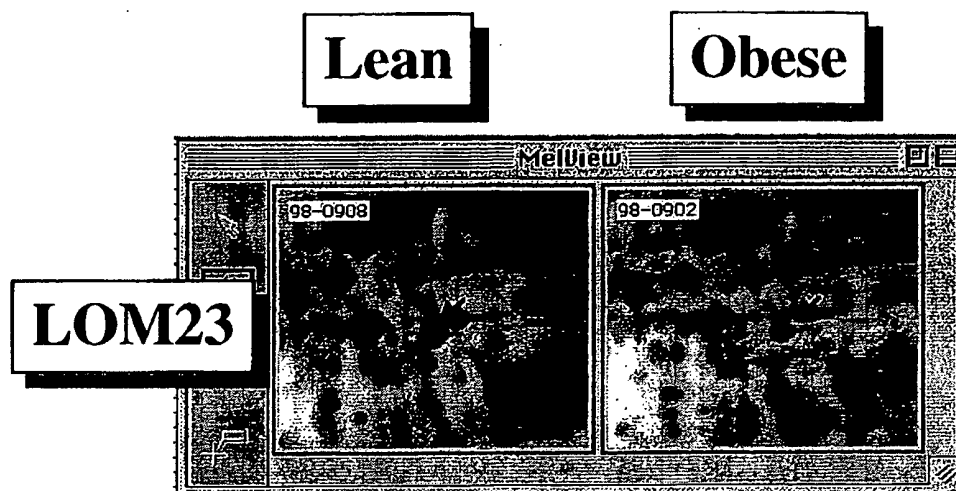


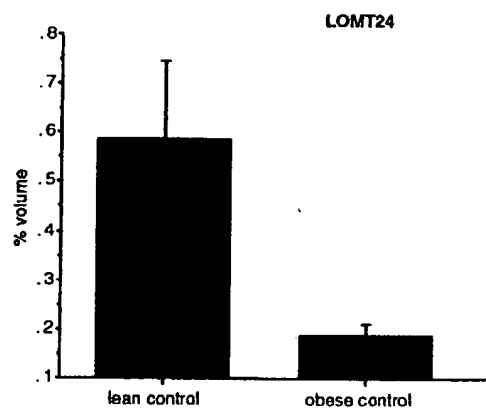
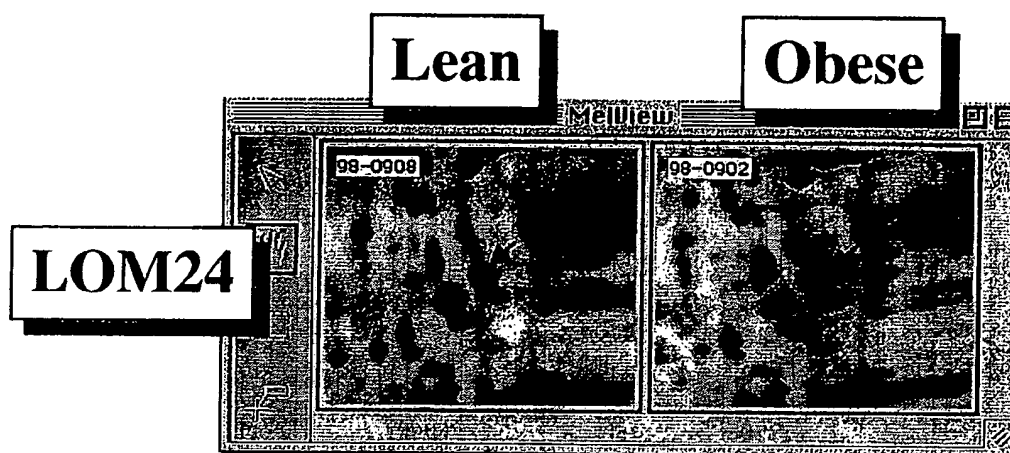
Figure 6

Figure 6 continued

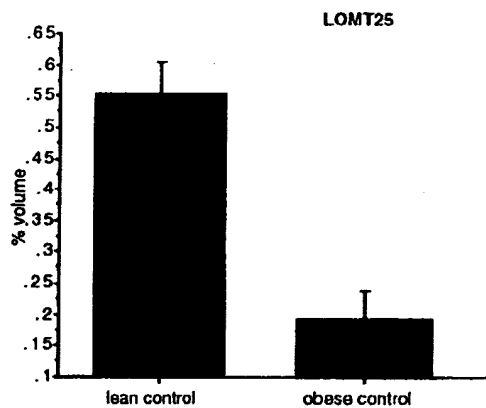
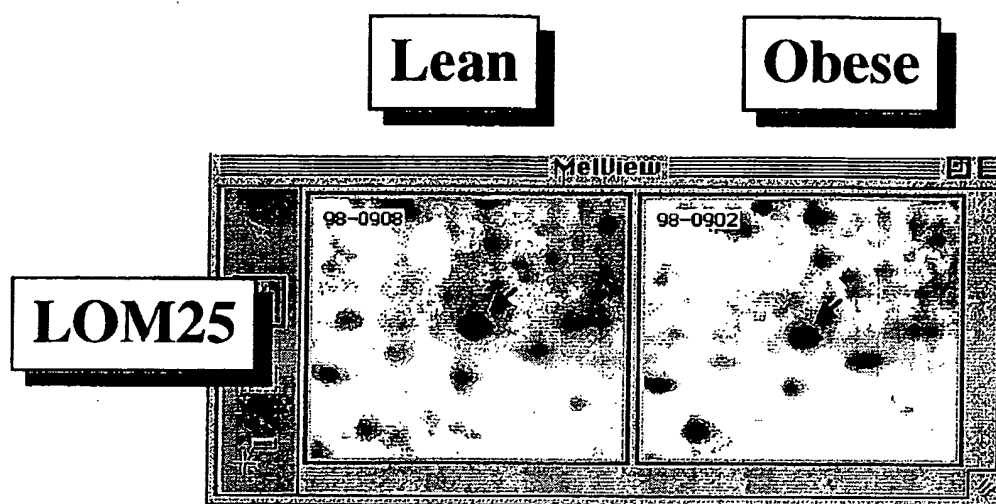


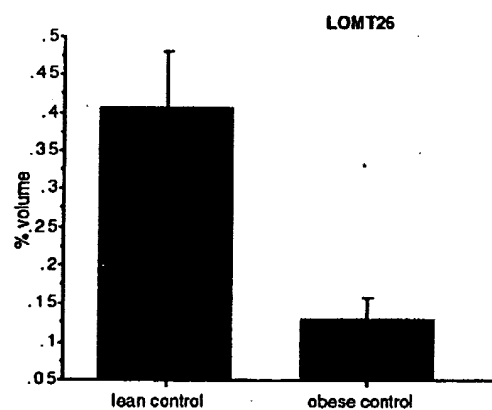
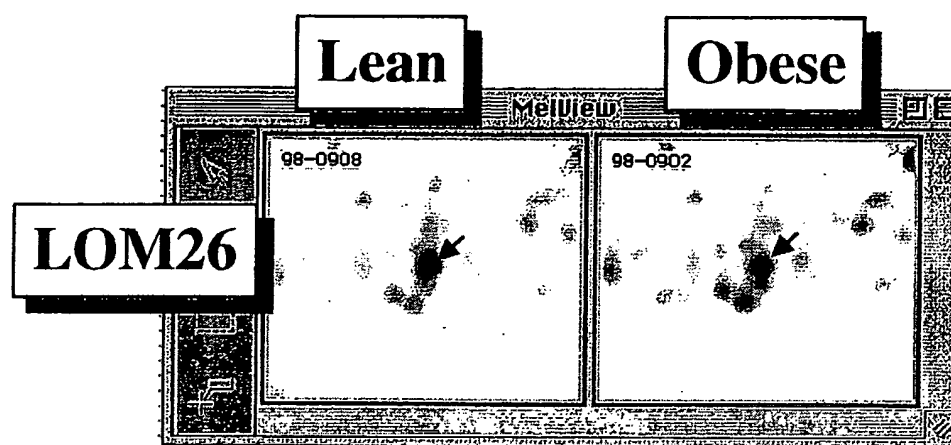
Figure 7

Figure 7 continued

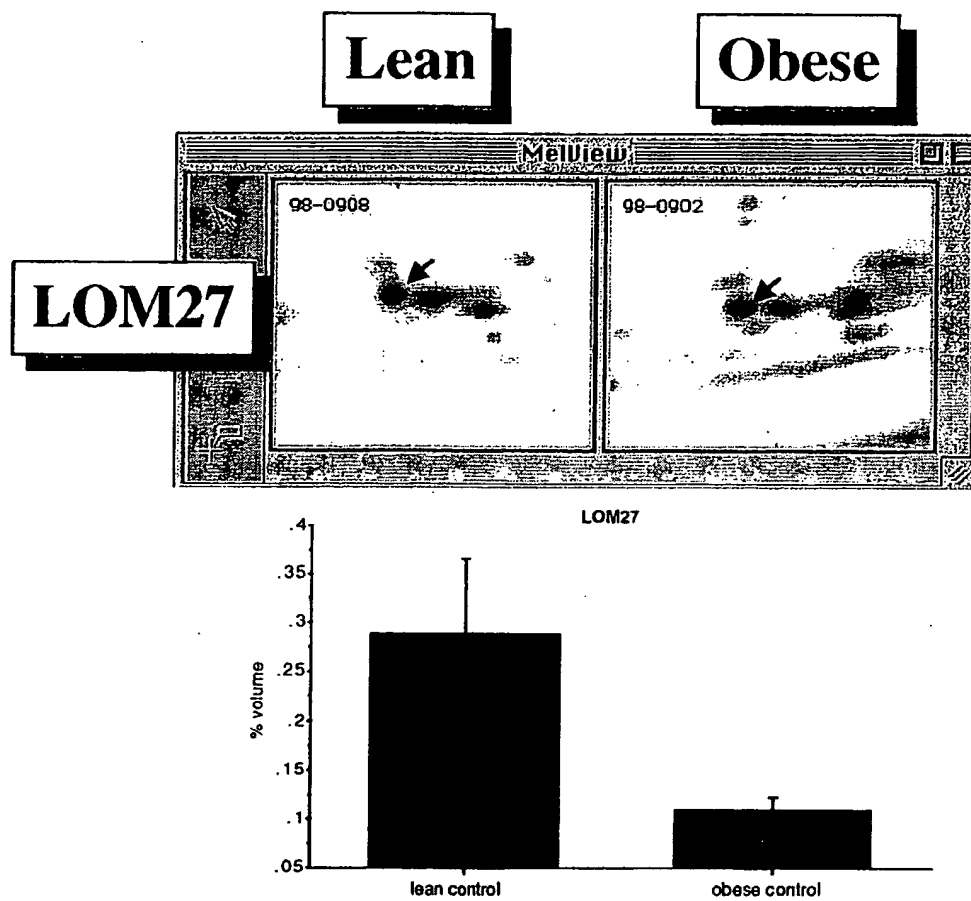


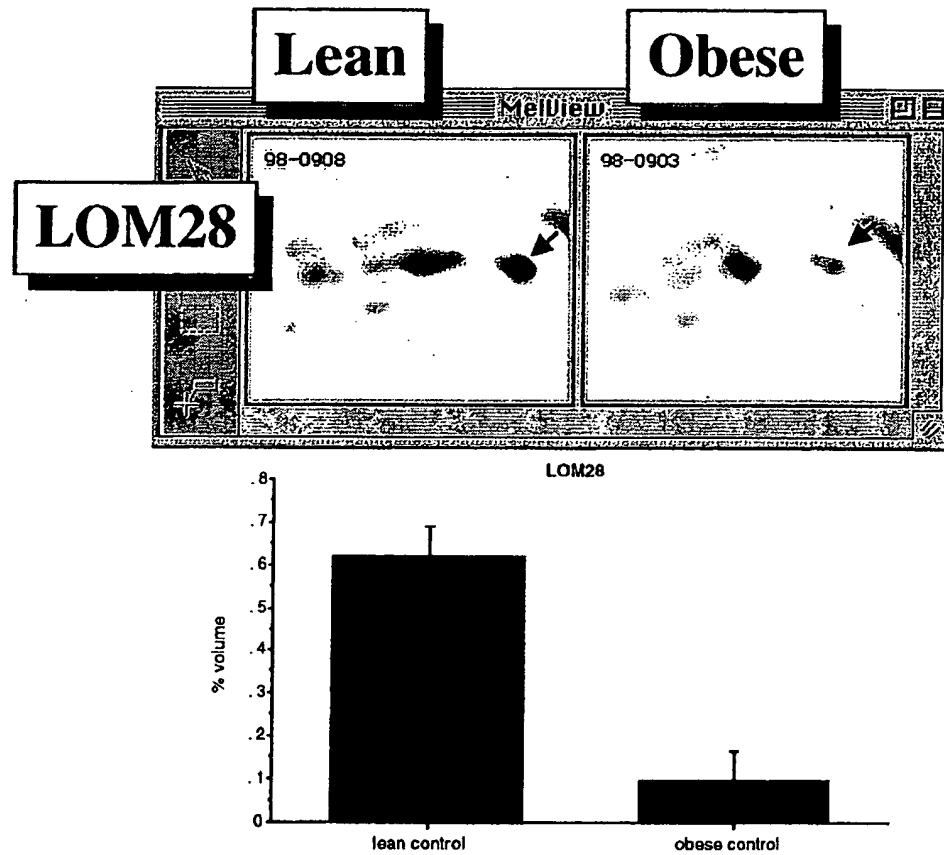
Figure 8

Figure 8 continued

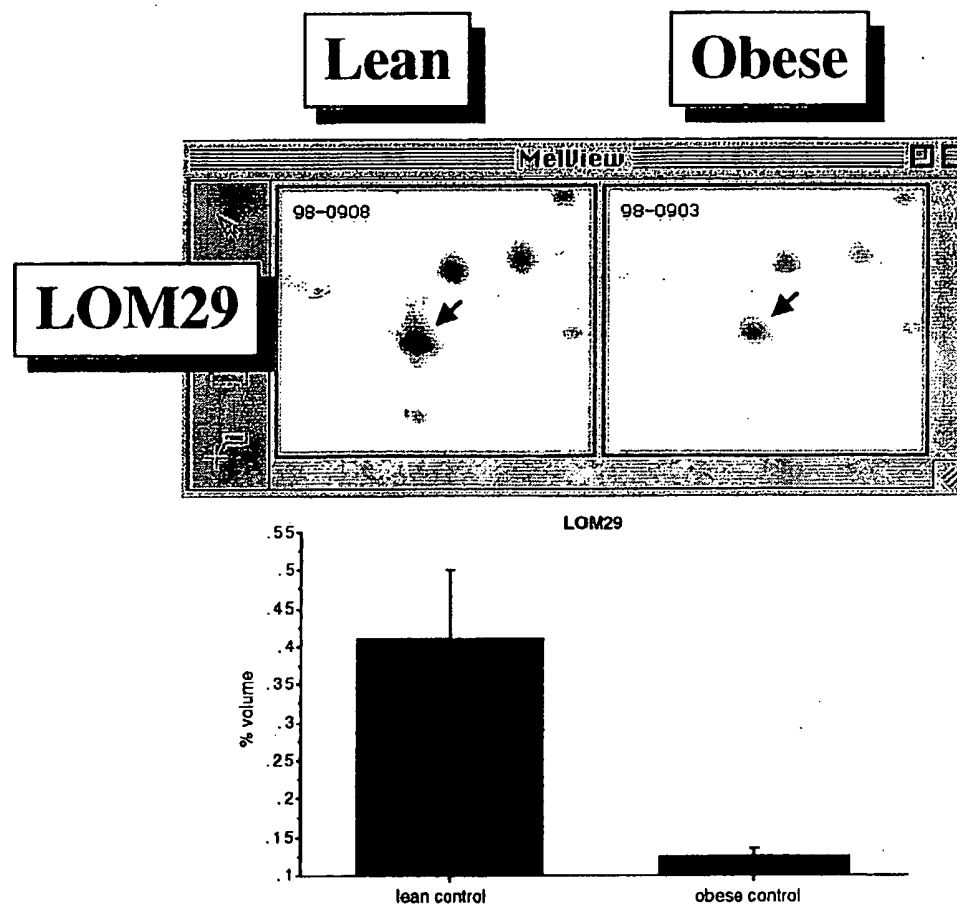


Figure 9

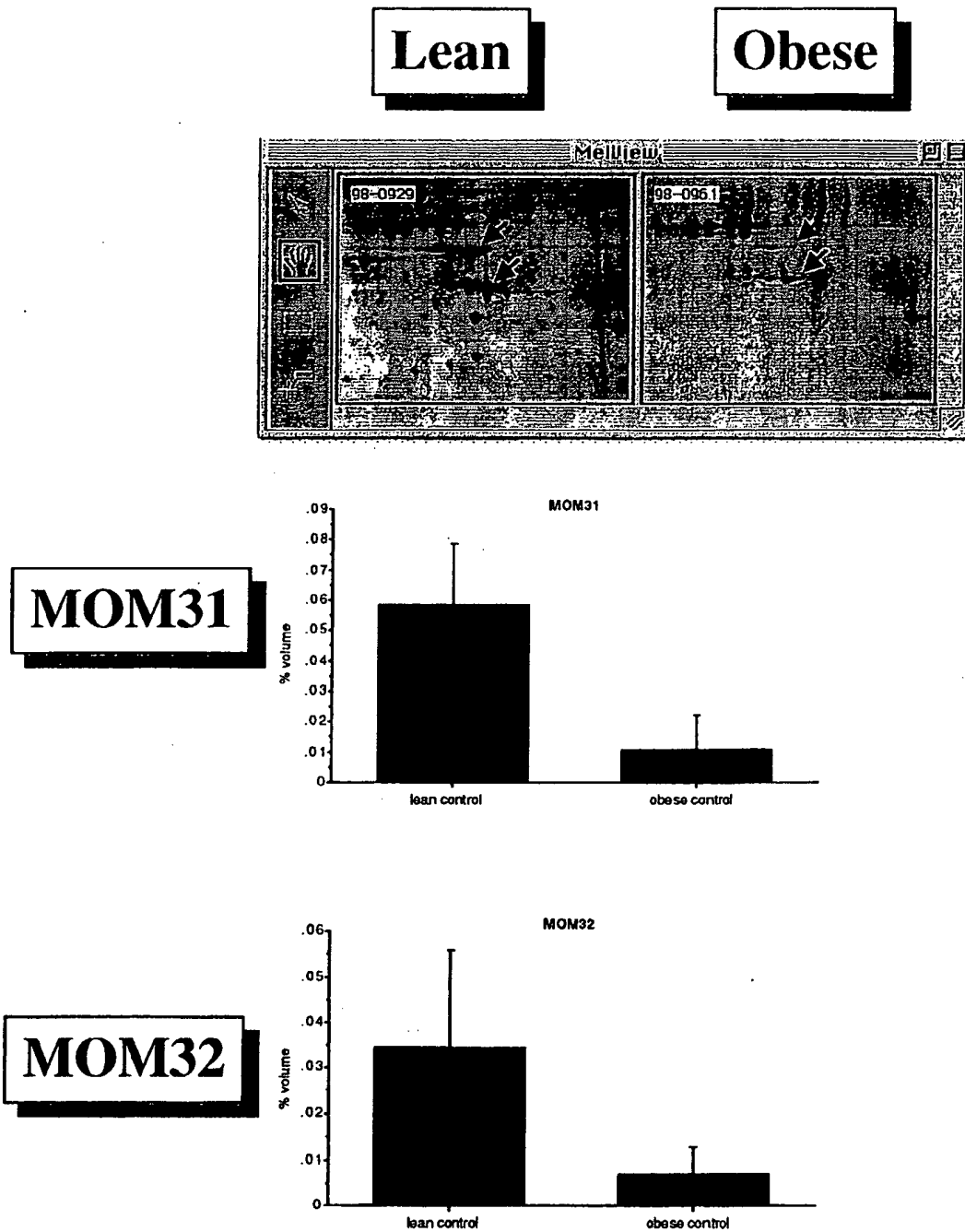


Figure 9 continued

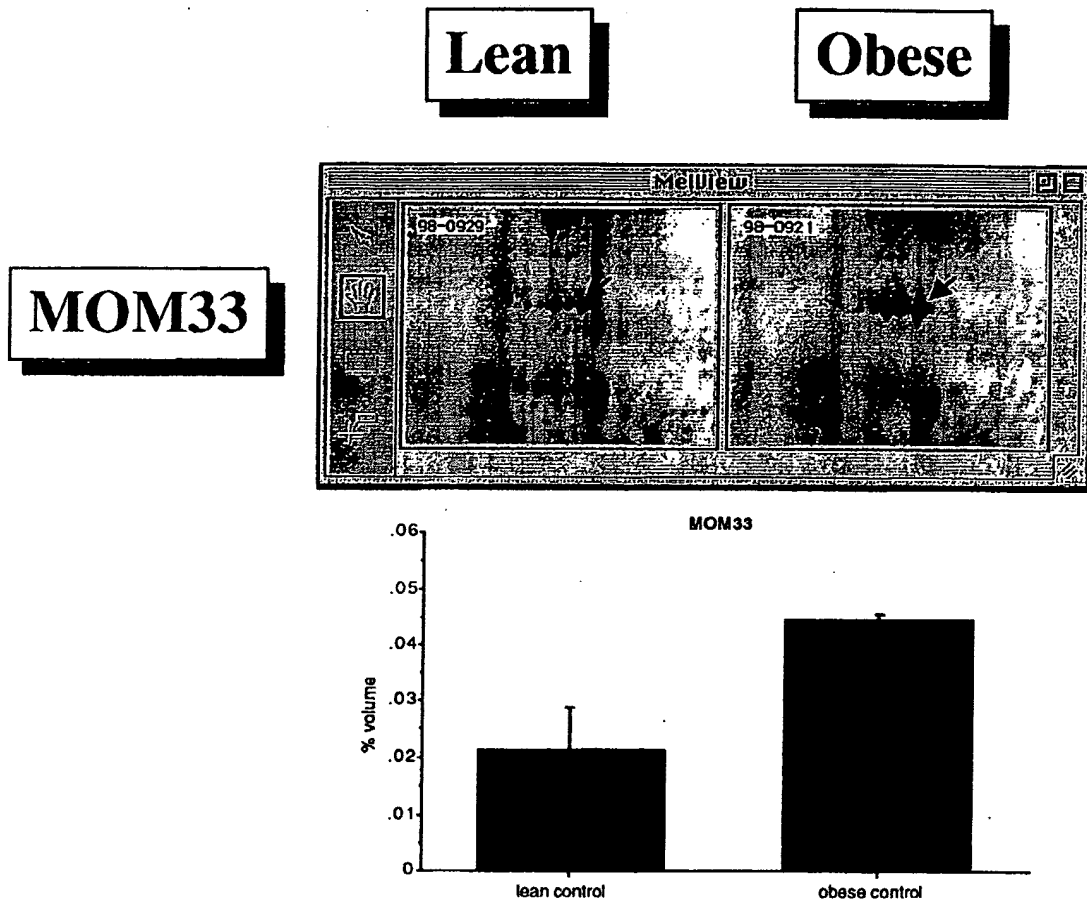


Figure 10

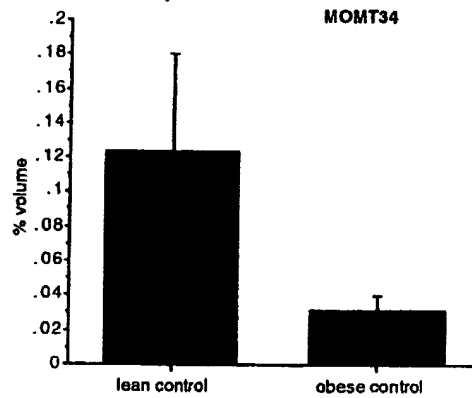
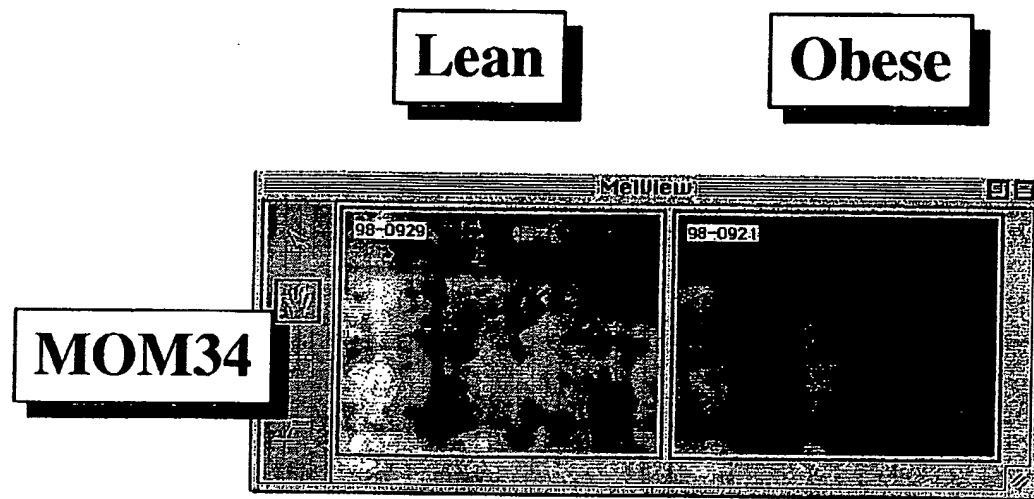


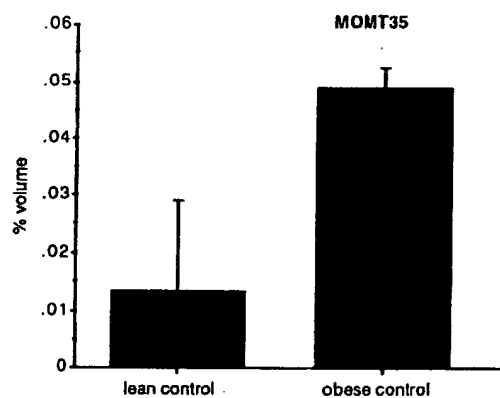
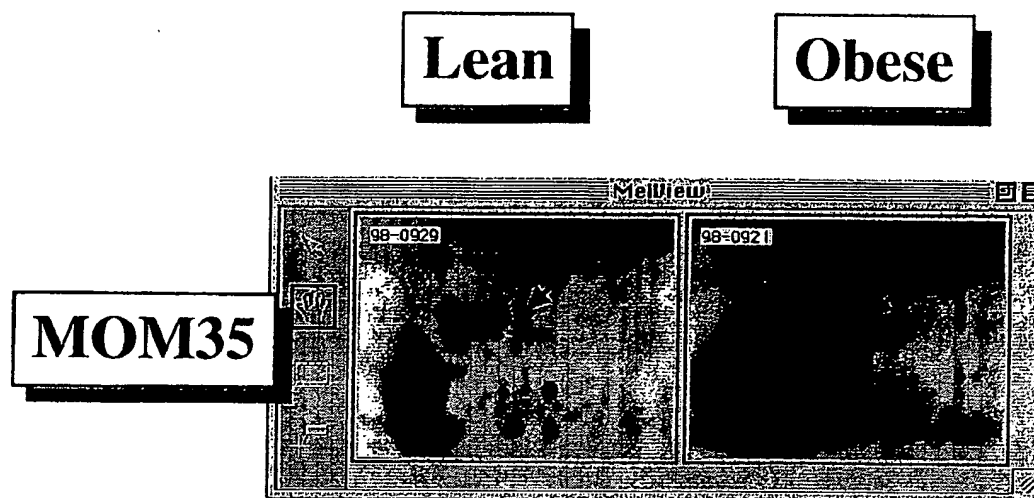
Figure 10 continued

Figure 11

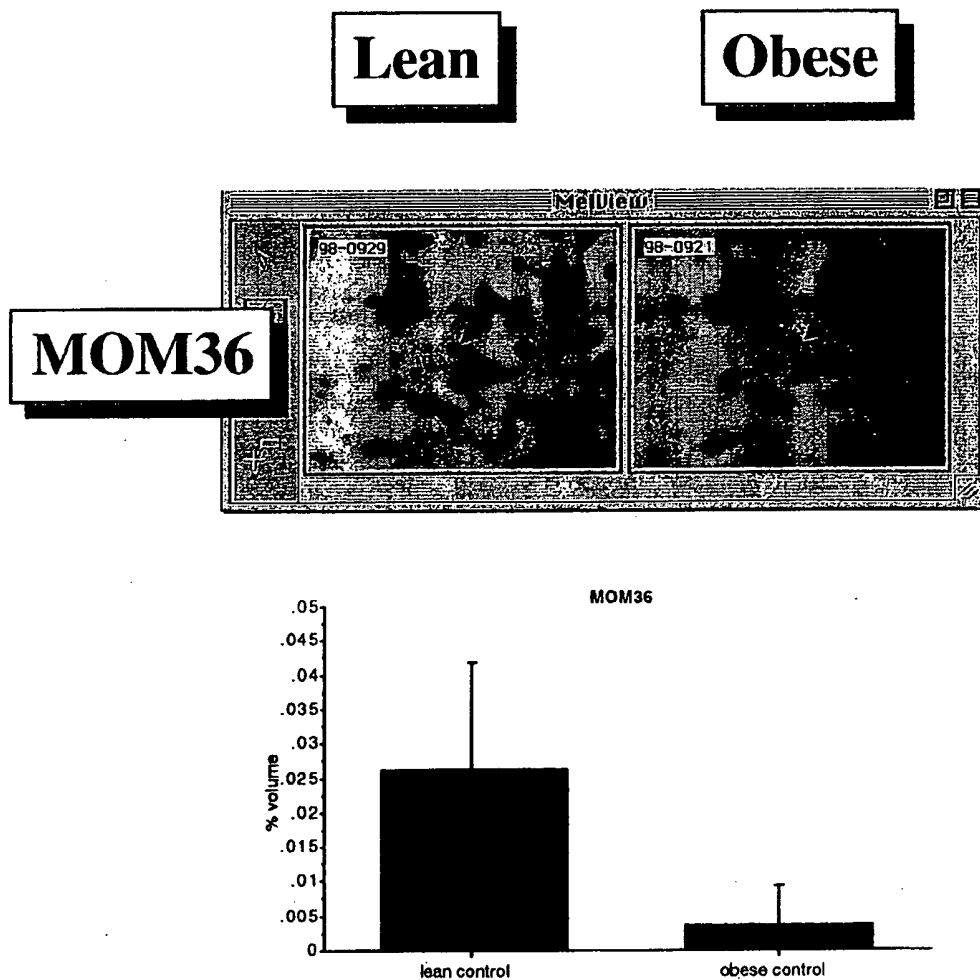


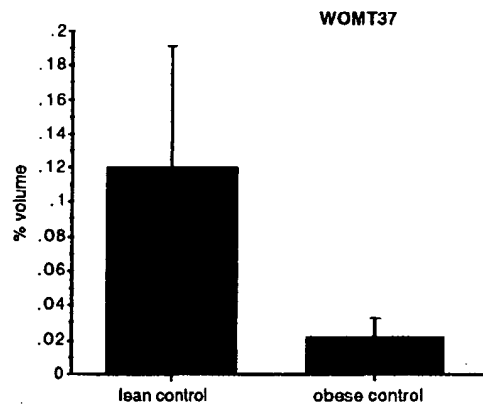
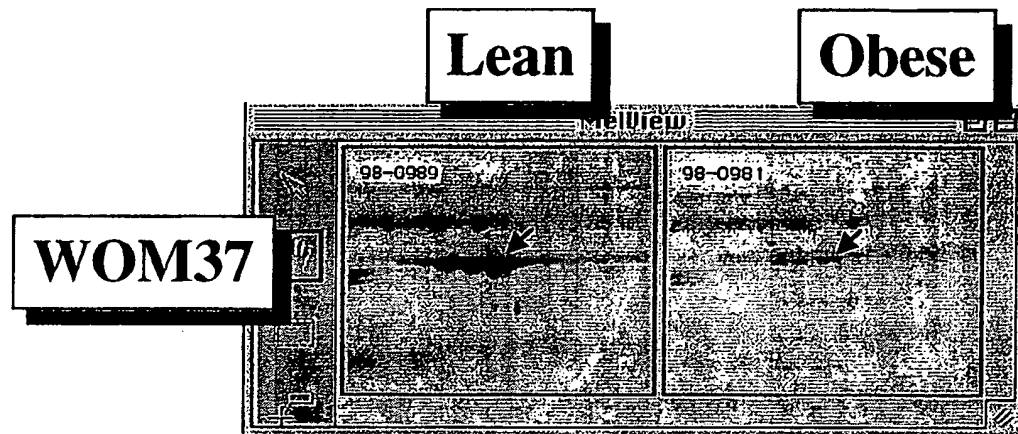
Figure 12

Figure 12 continued

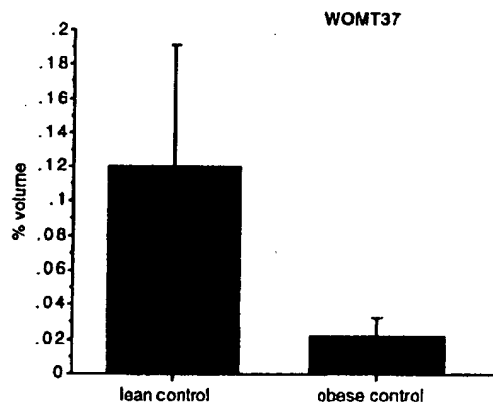
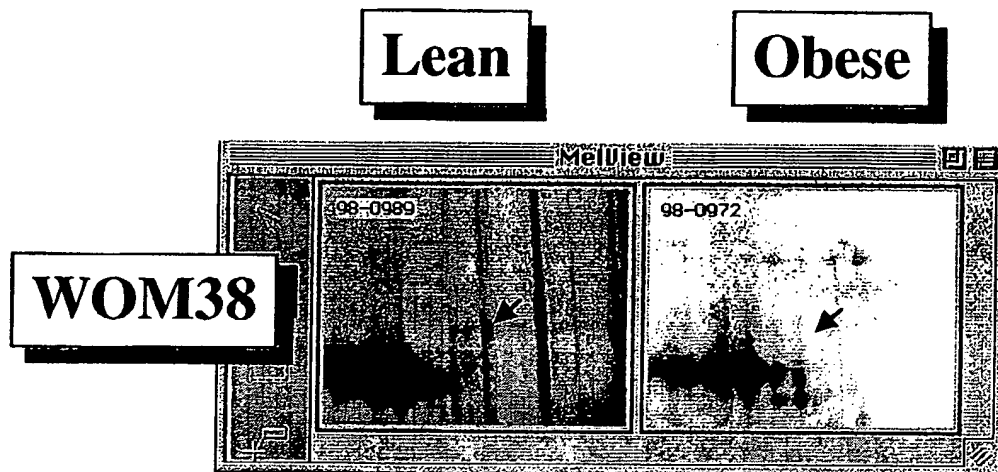


Figure 13

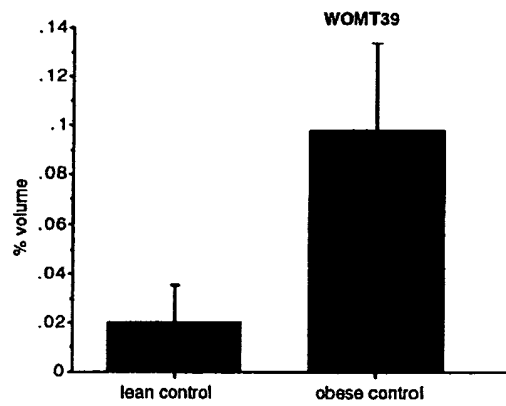
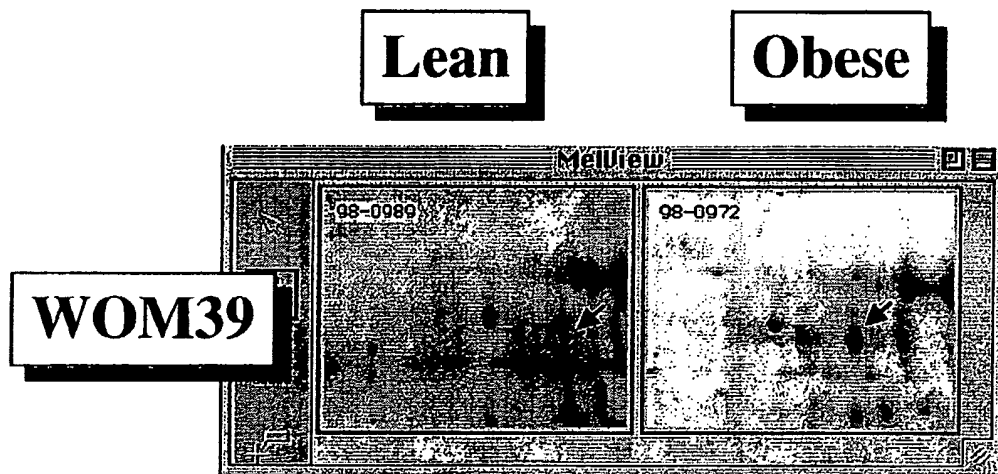


Figure 13 continued

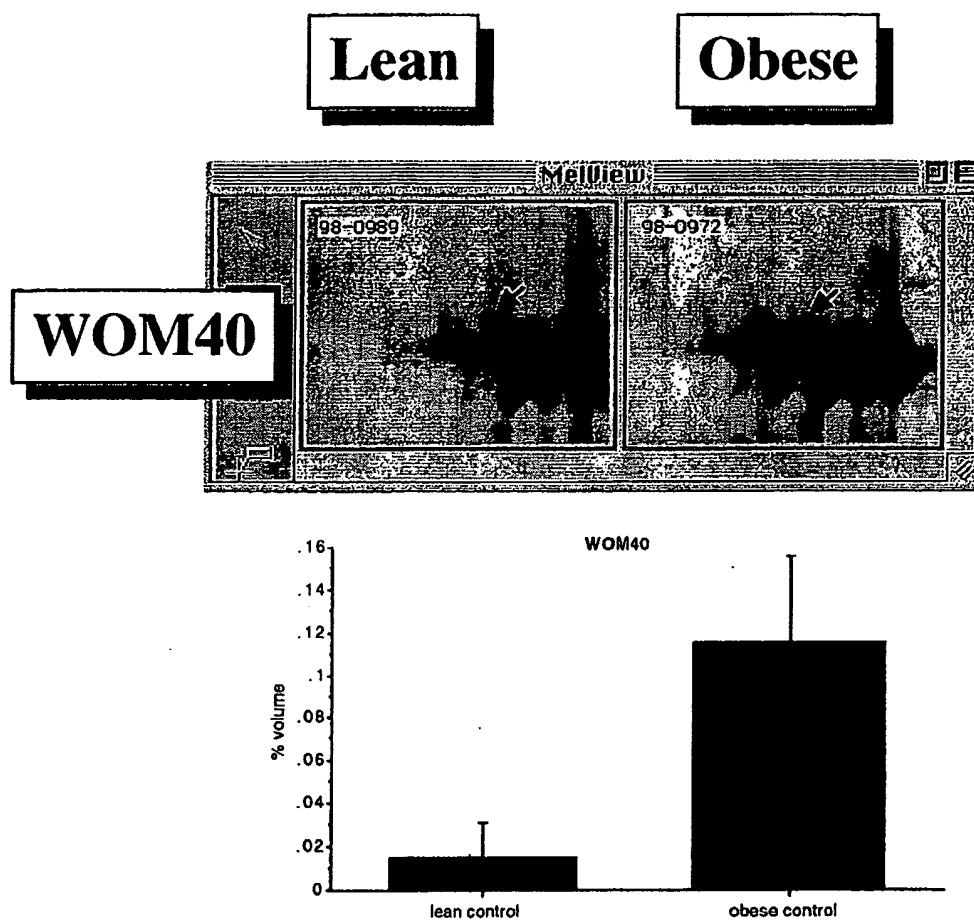


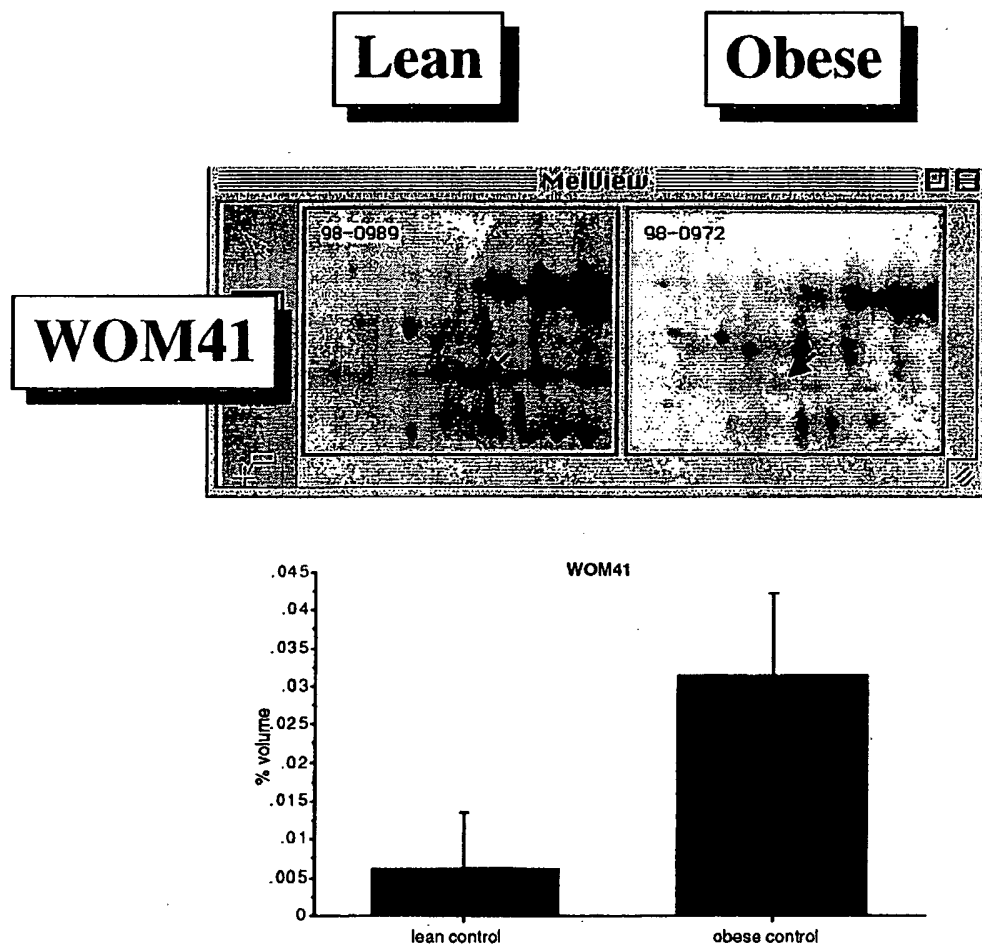
Figure 14

Figure 14 continued

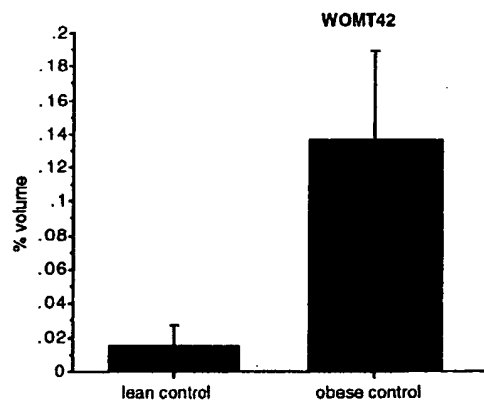
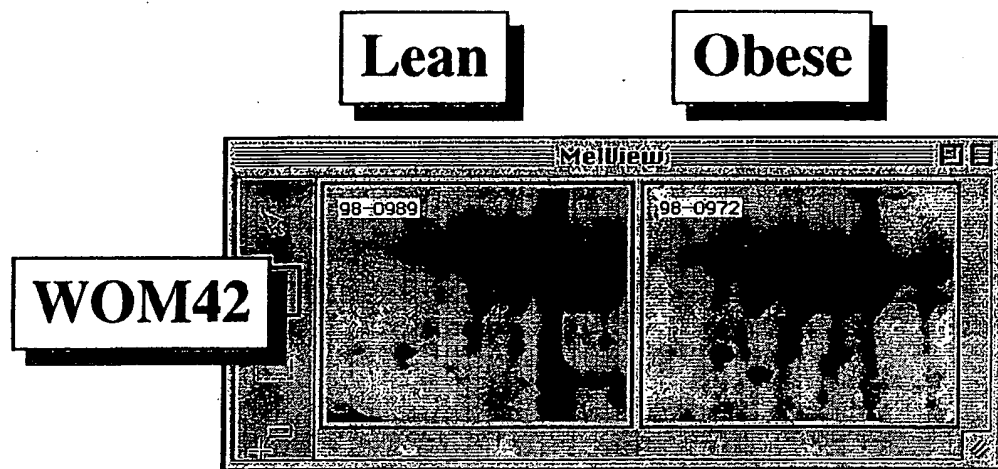


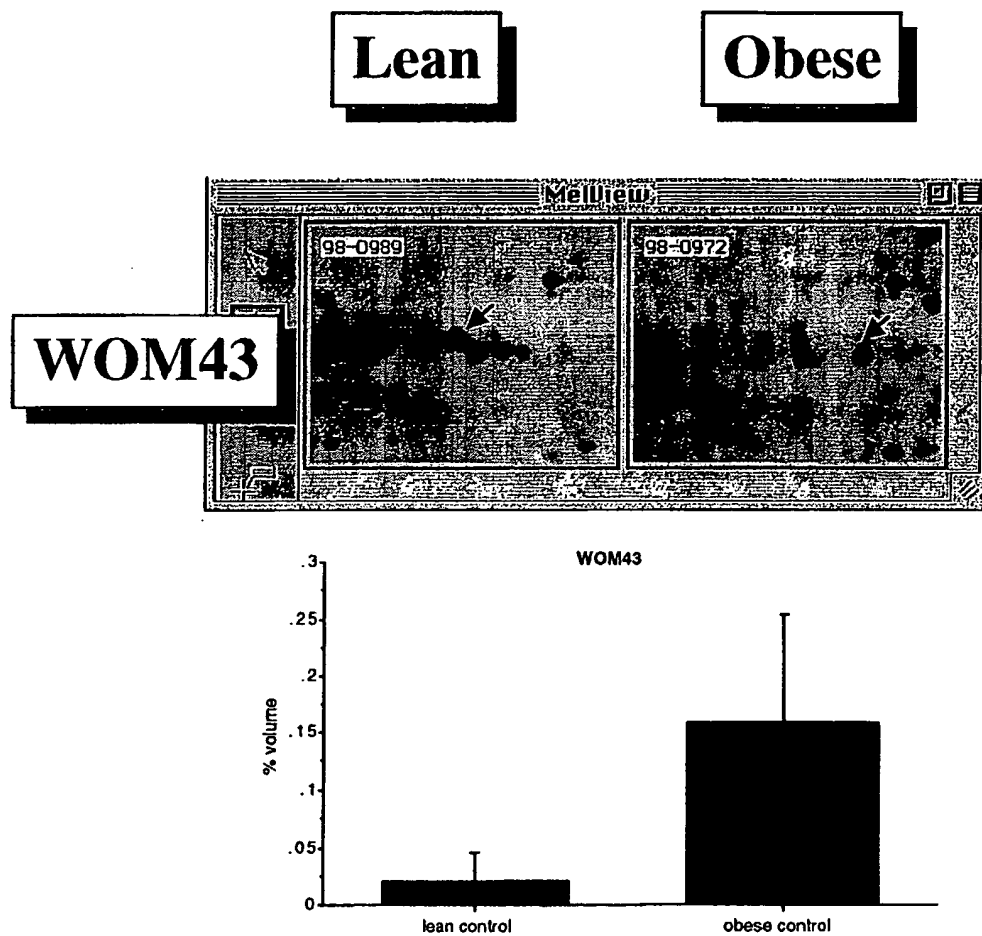
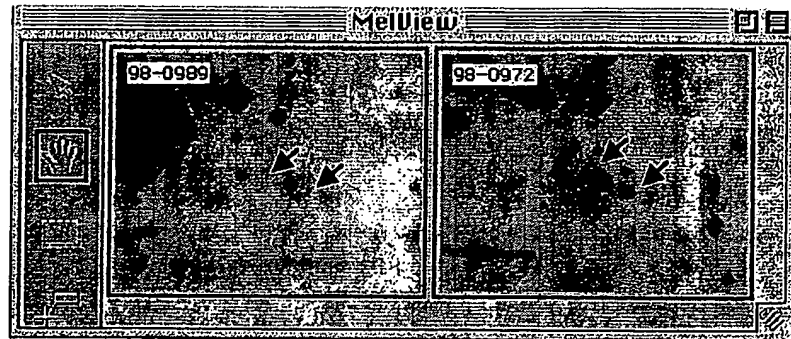
Figure 15

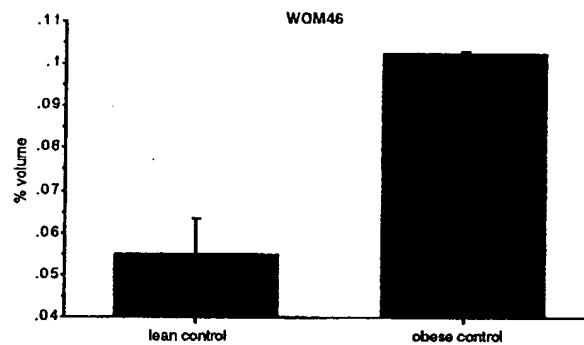
Figure 16

Lean

Obese



WOM46



WOM47

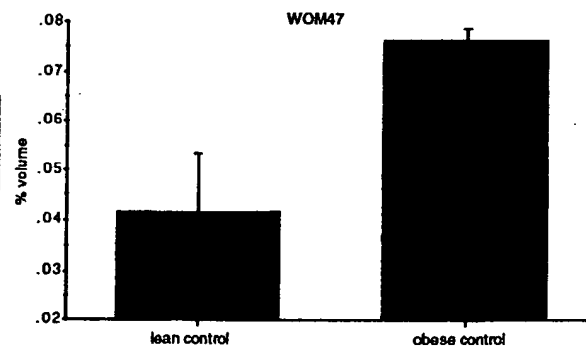
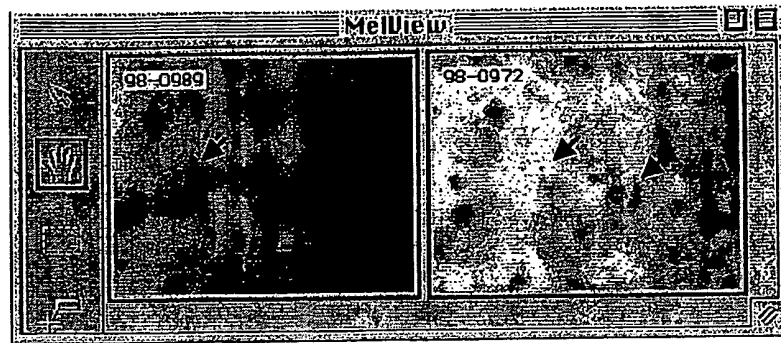


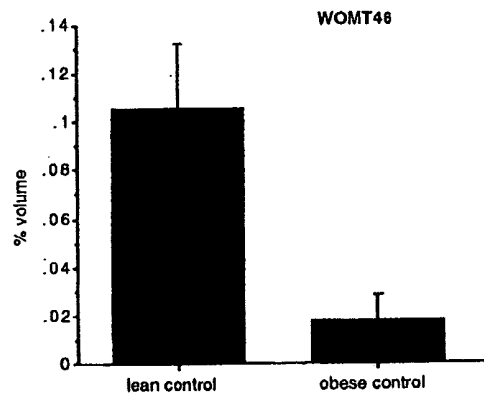
Figure 16 continued

Lean

Obese



WOM48



WOM49

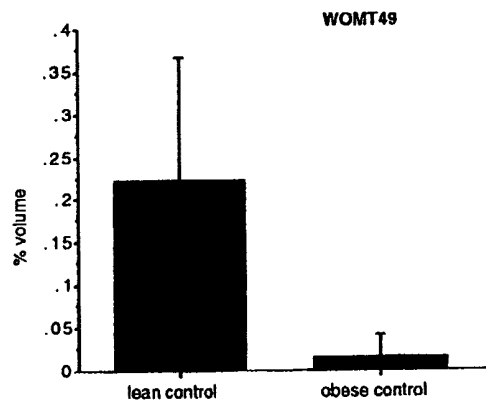


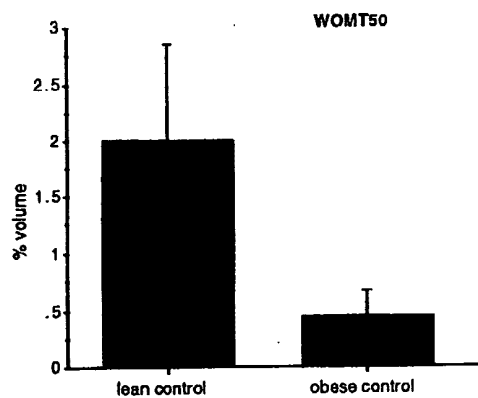
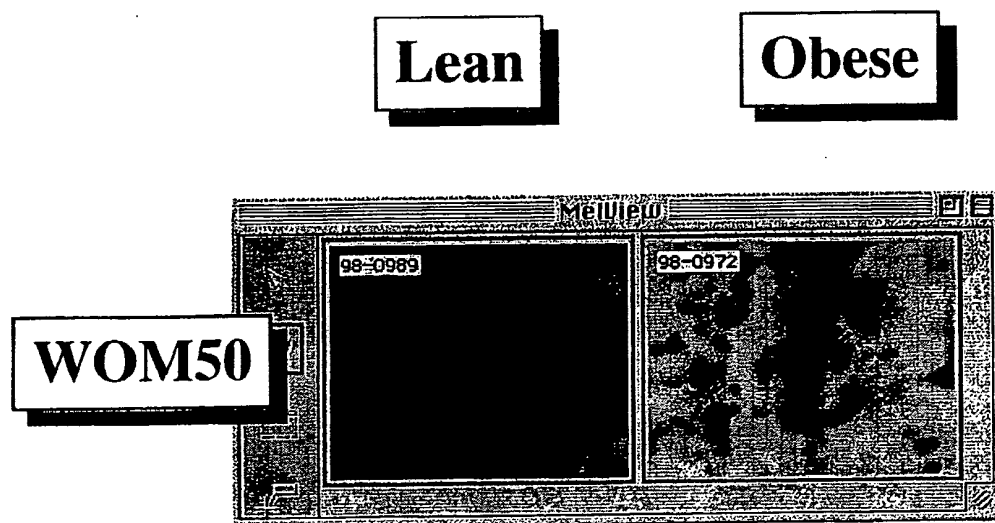
Figure 17

Figure 17 continued

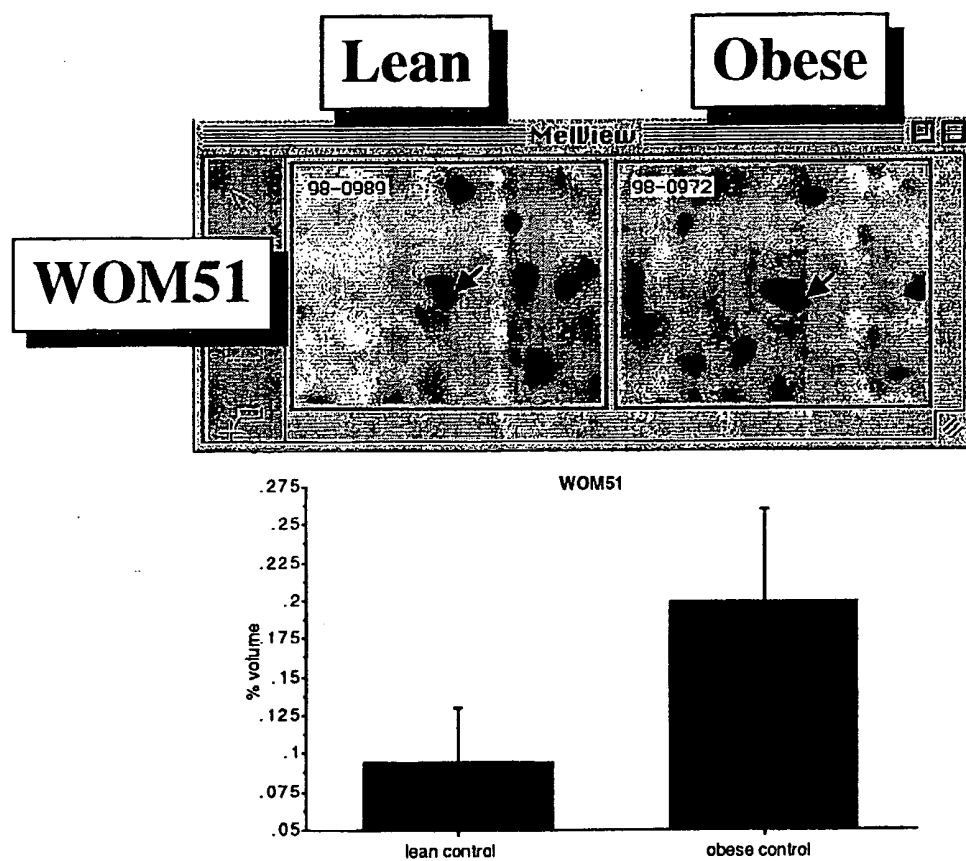


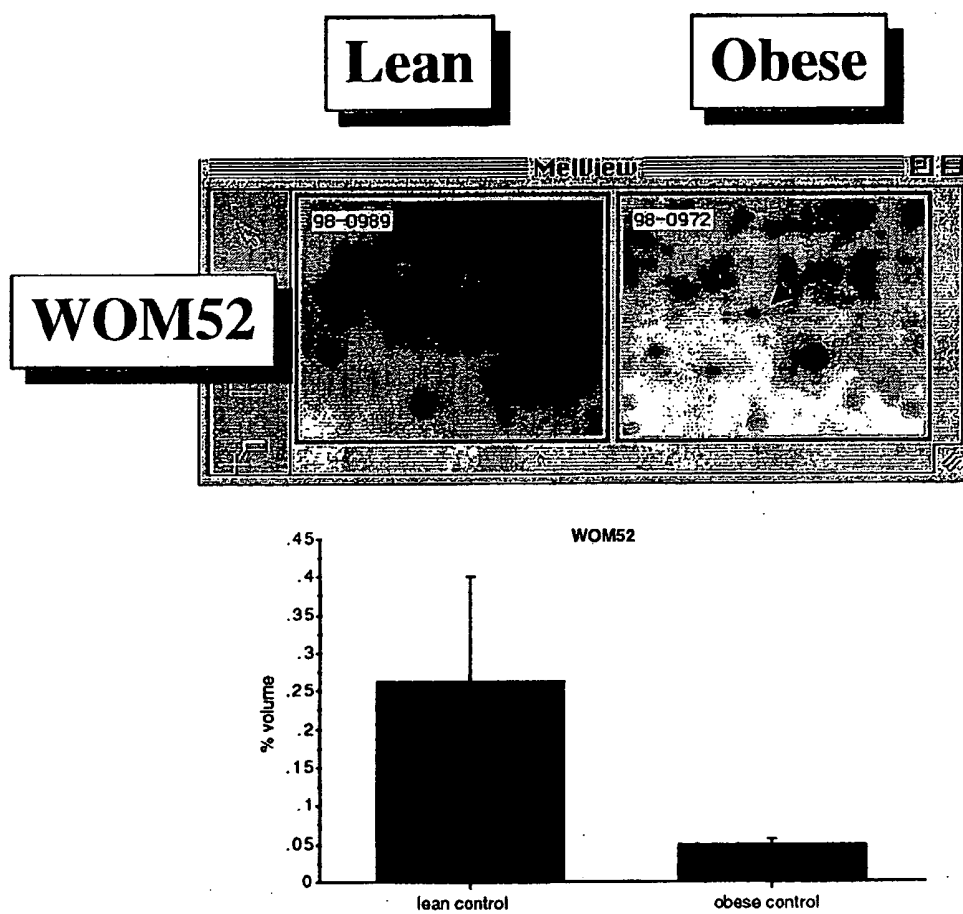
Figure 18

Figure 18 continued

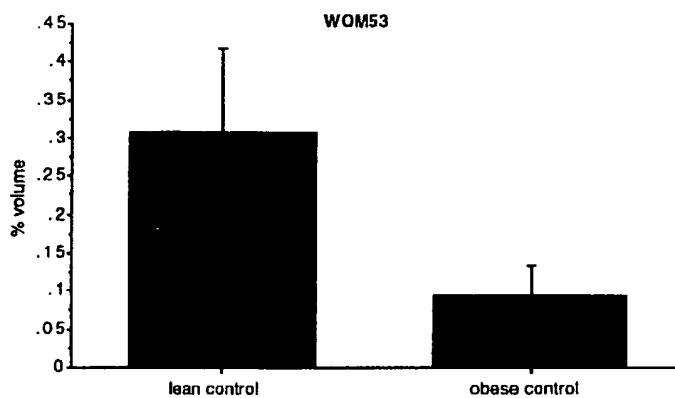
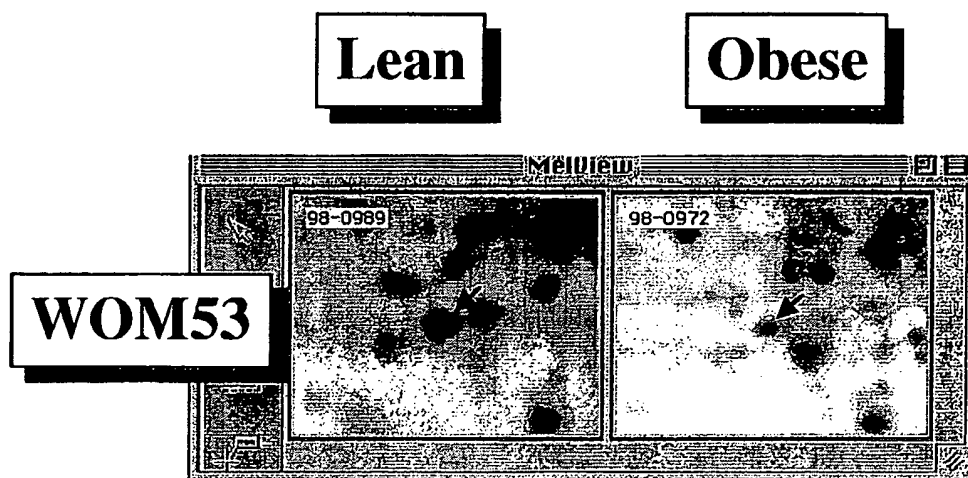


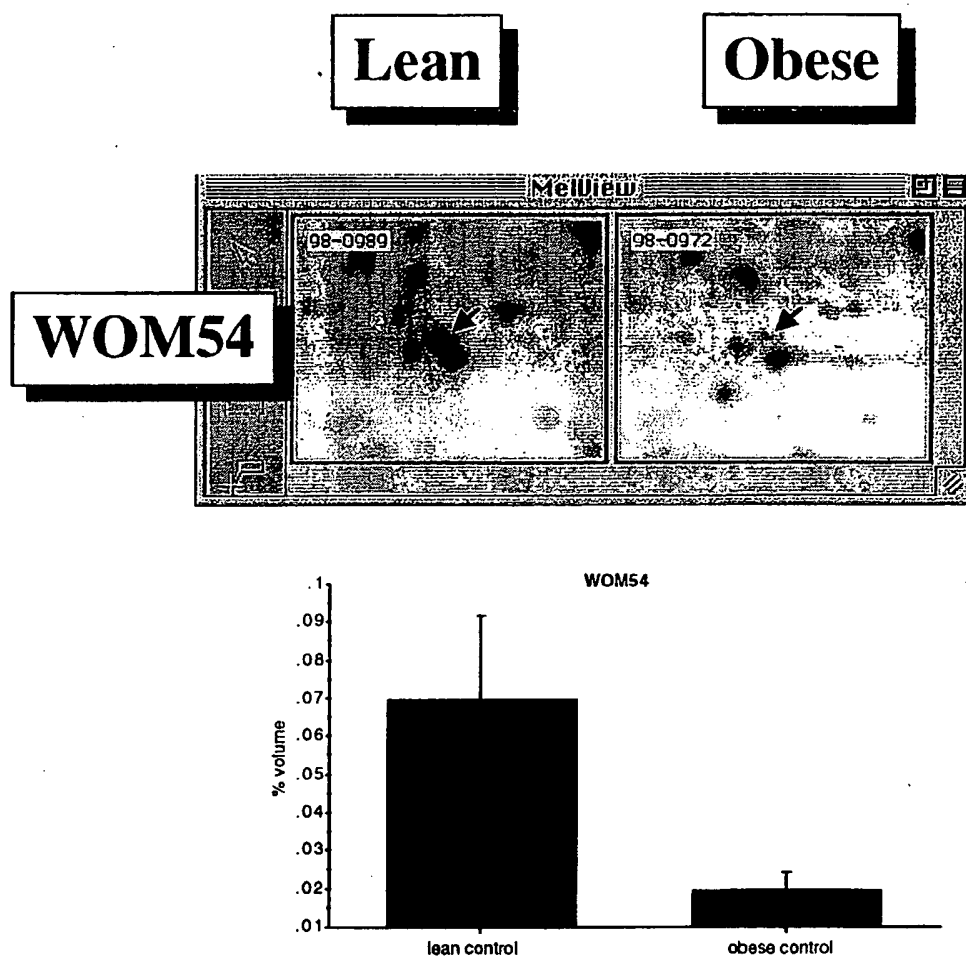
Figure 19

Figure 19 continued

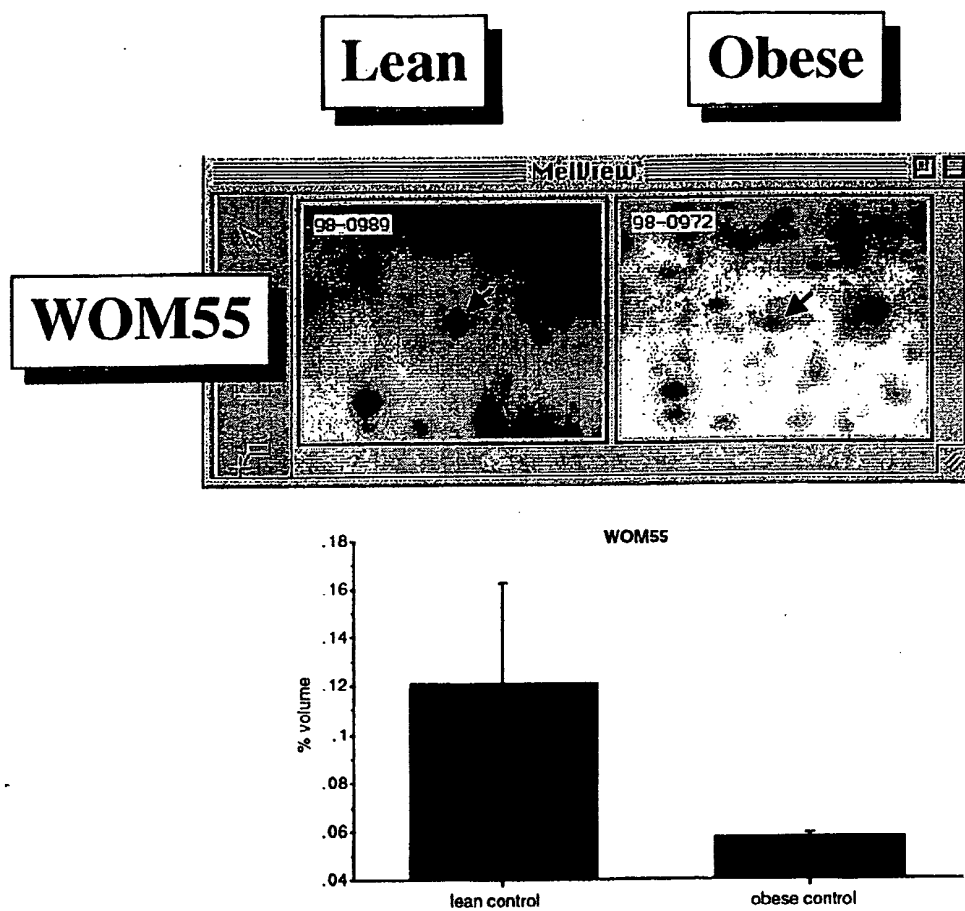


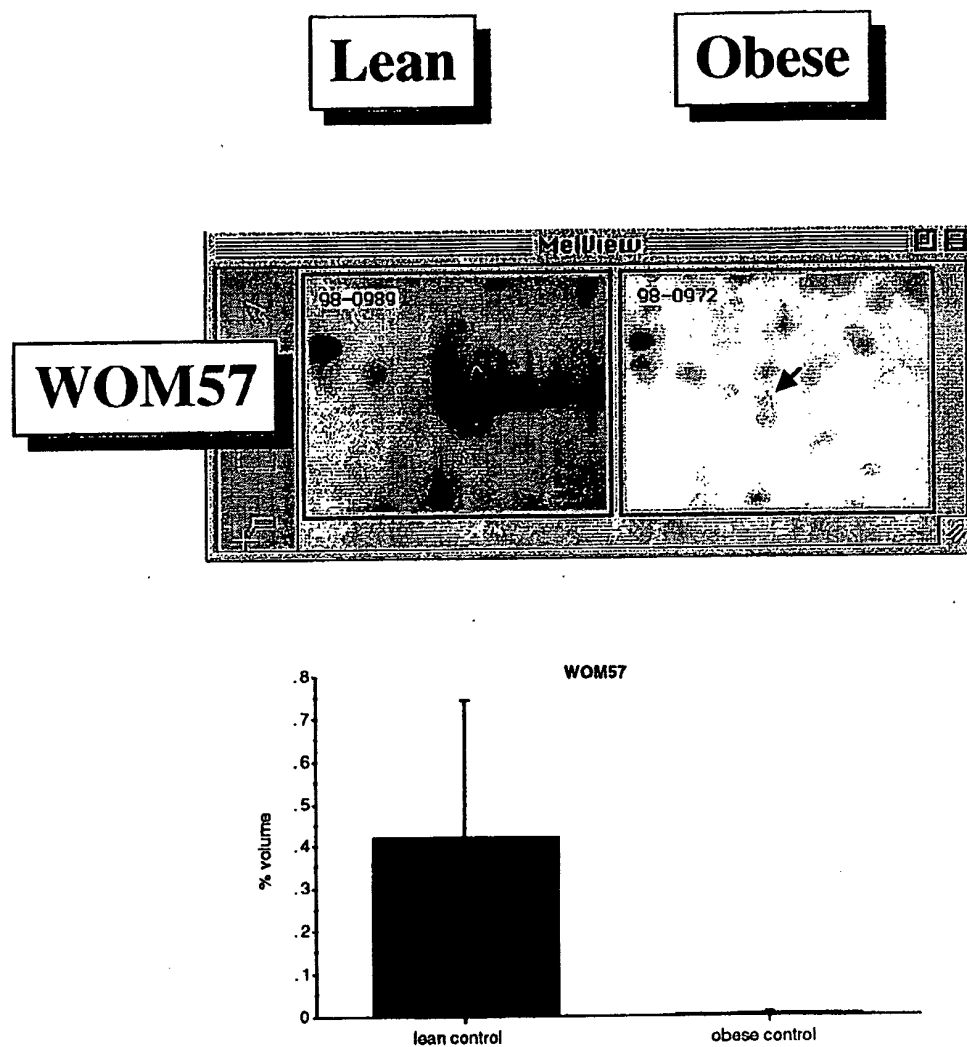
Figure 20

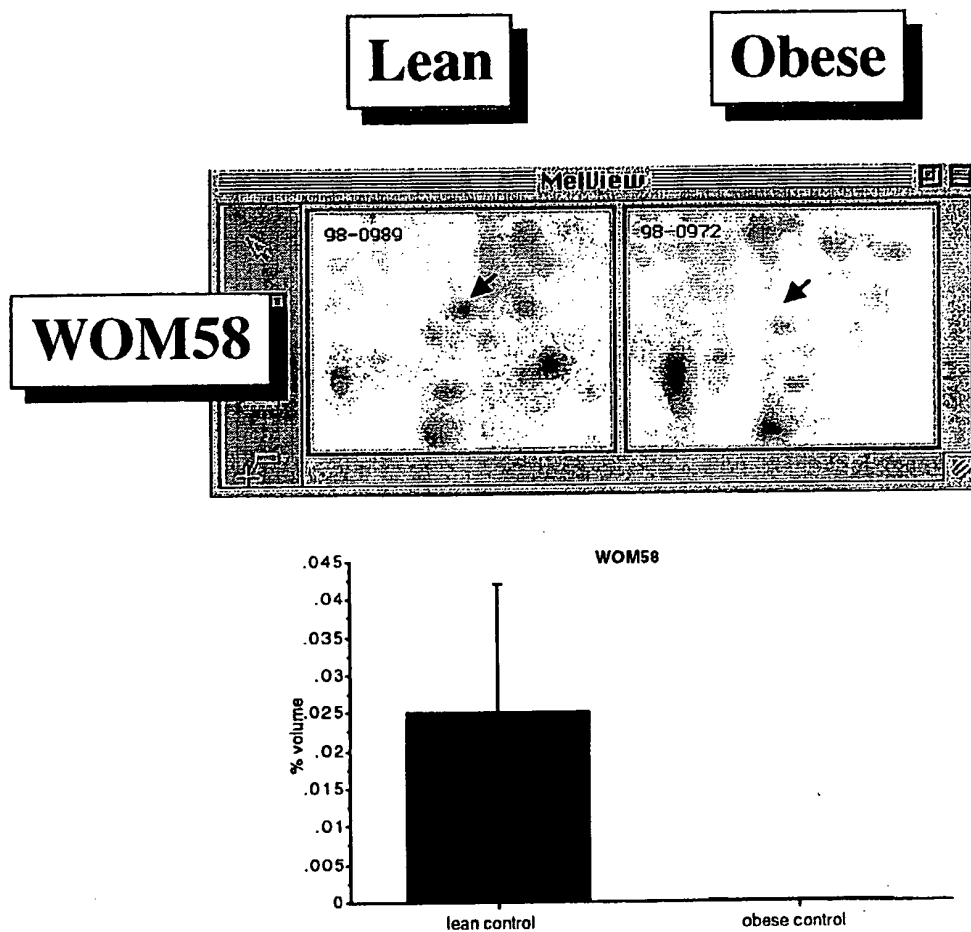
Figure 21

Figure 21 continued

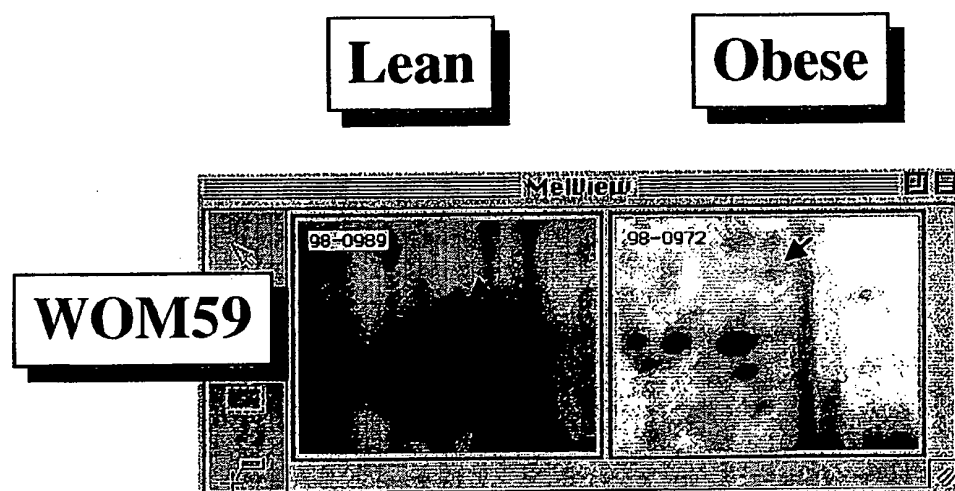


Figure 22

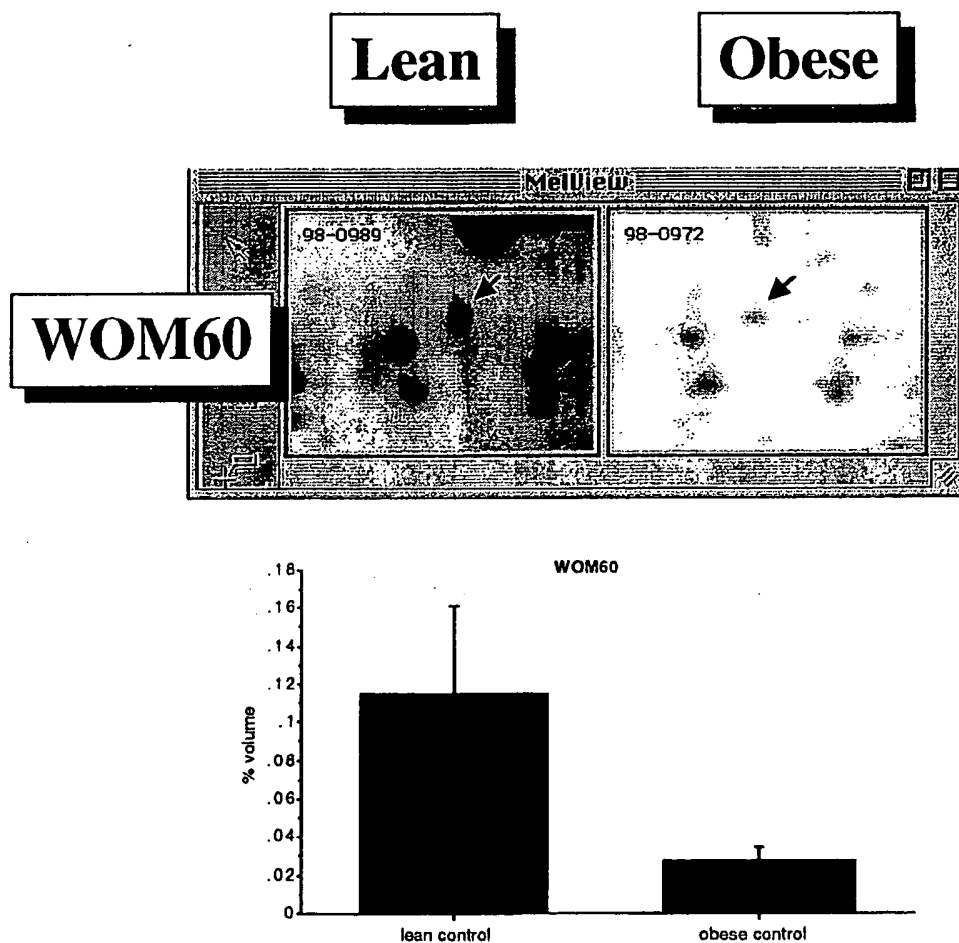


Figure 22 continued

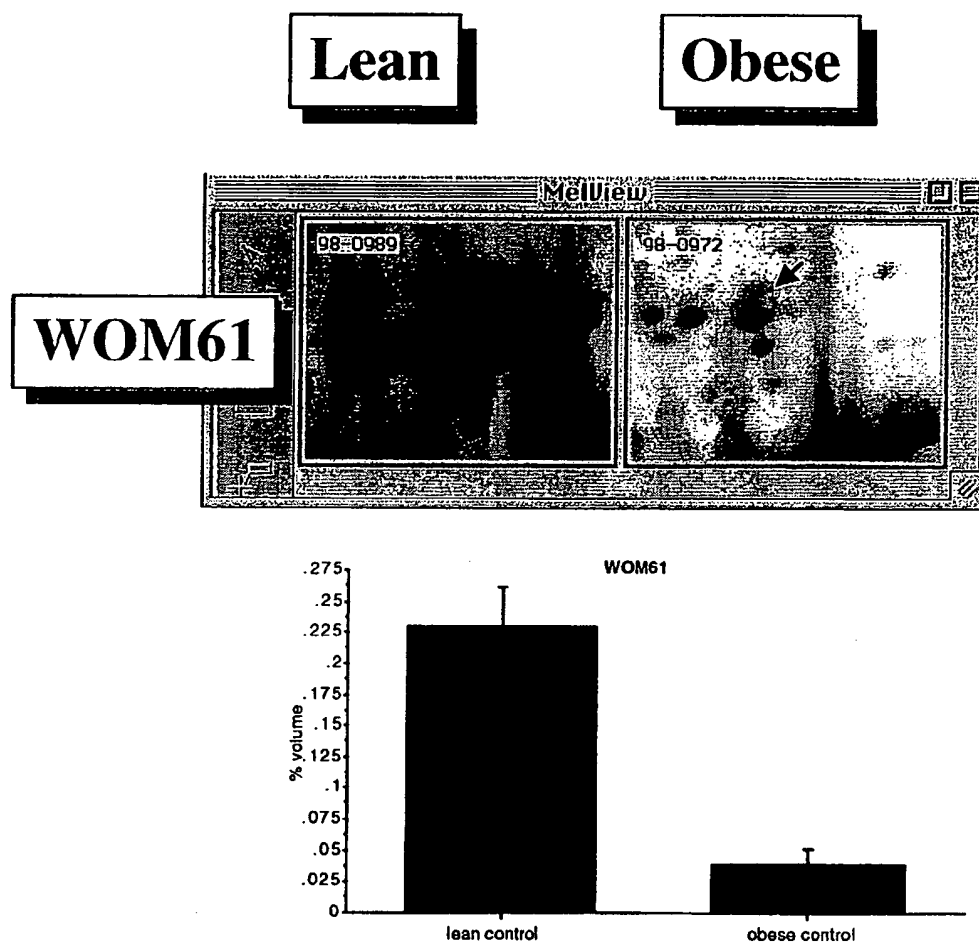


Figure 23

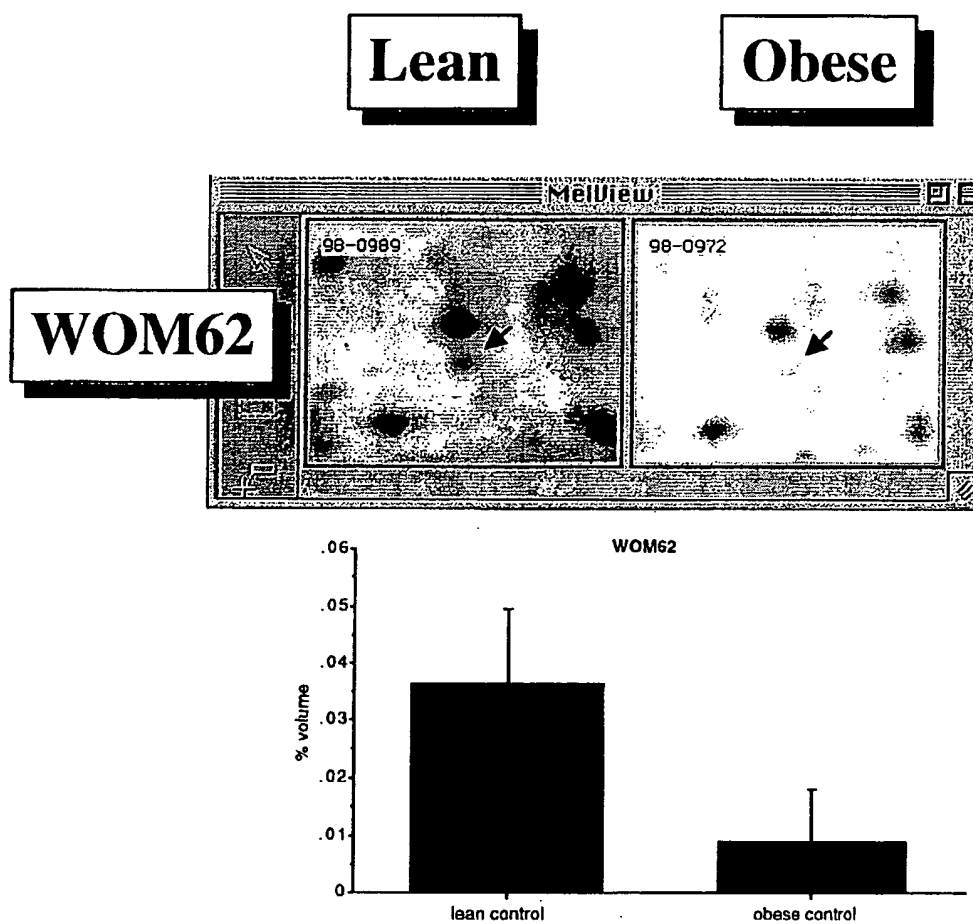
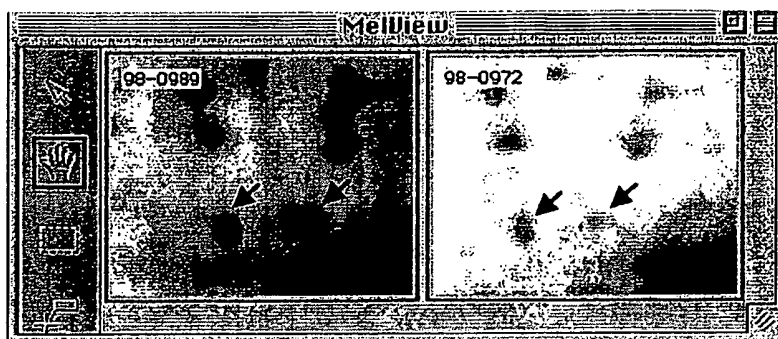


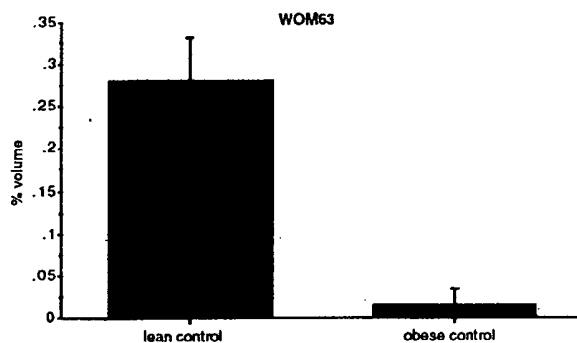
Figure 23 continued

Lean

Obese



WOM63



WOM64

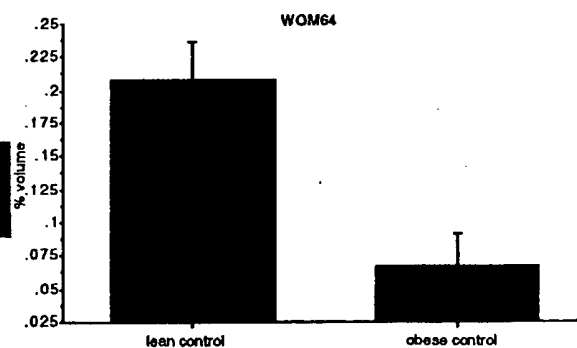


Figure 24

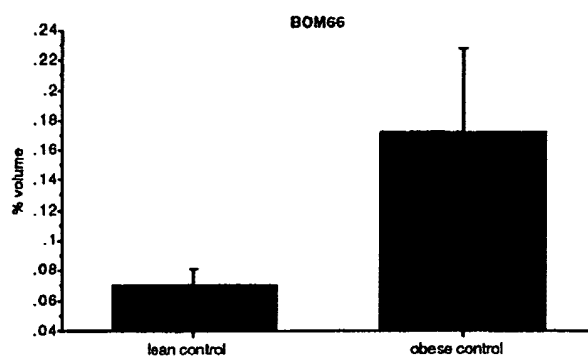
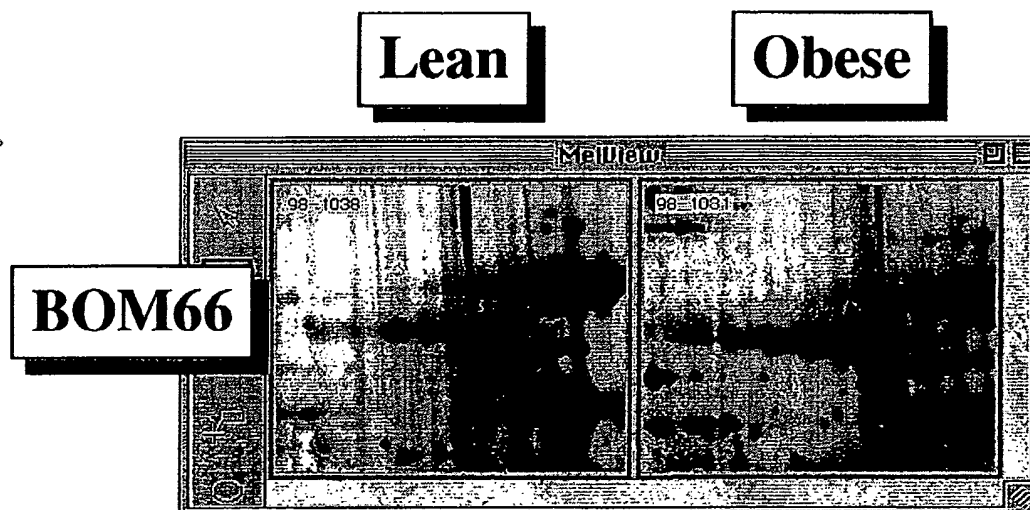


Figure 24 continued

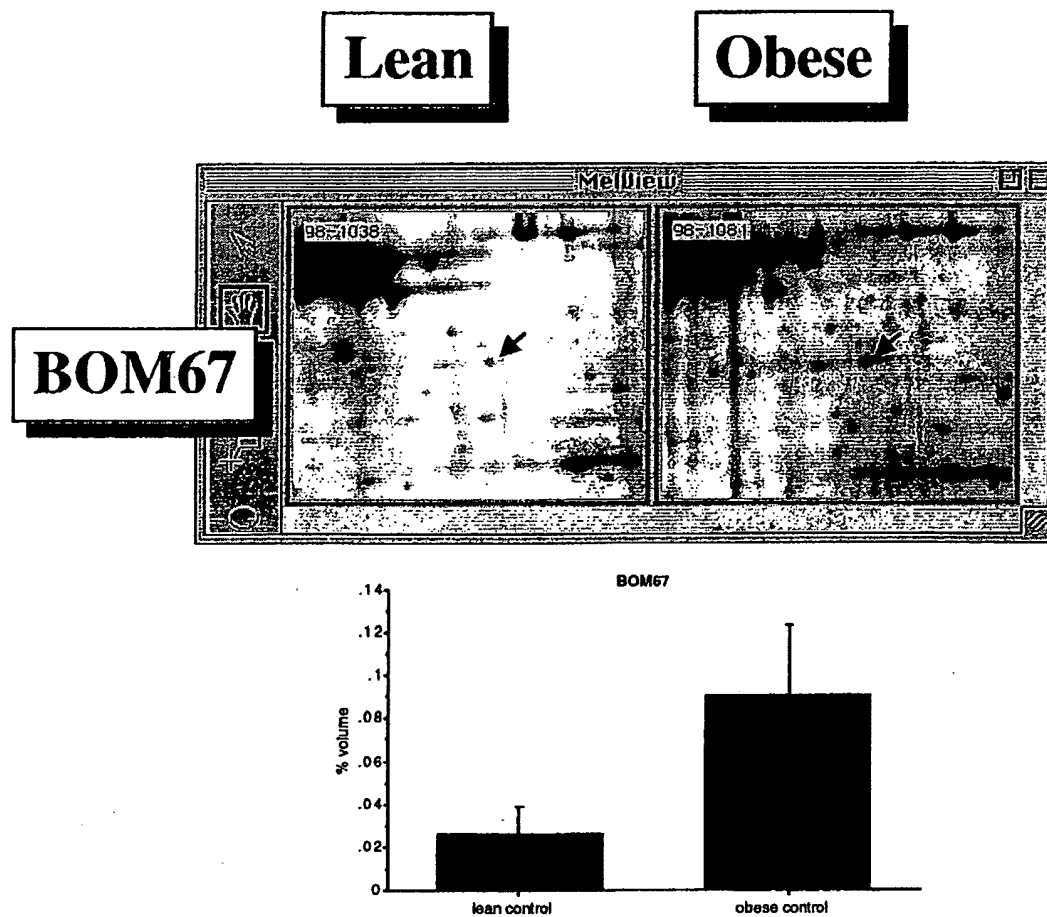


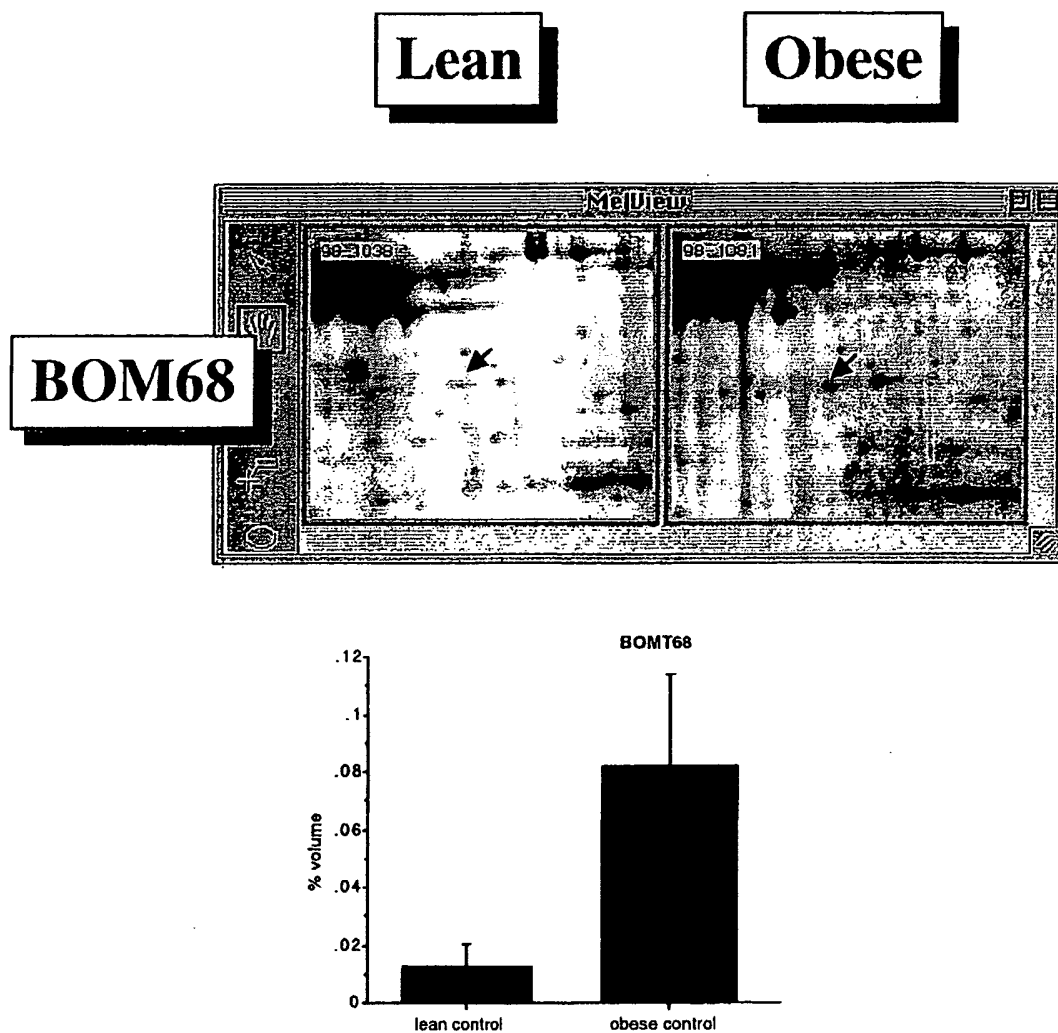
Figure 25

Figure 25 continued

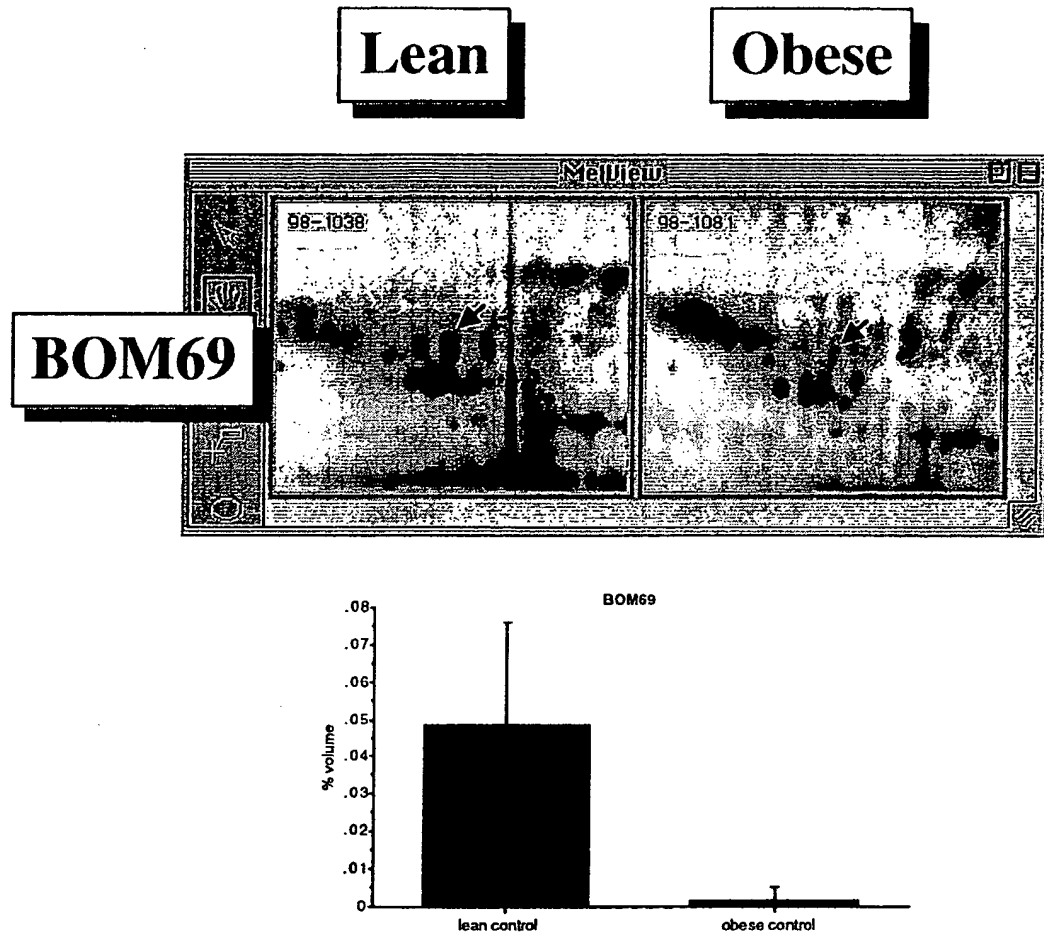


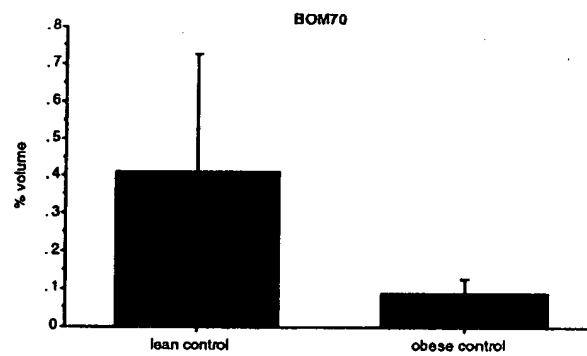
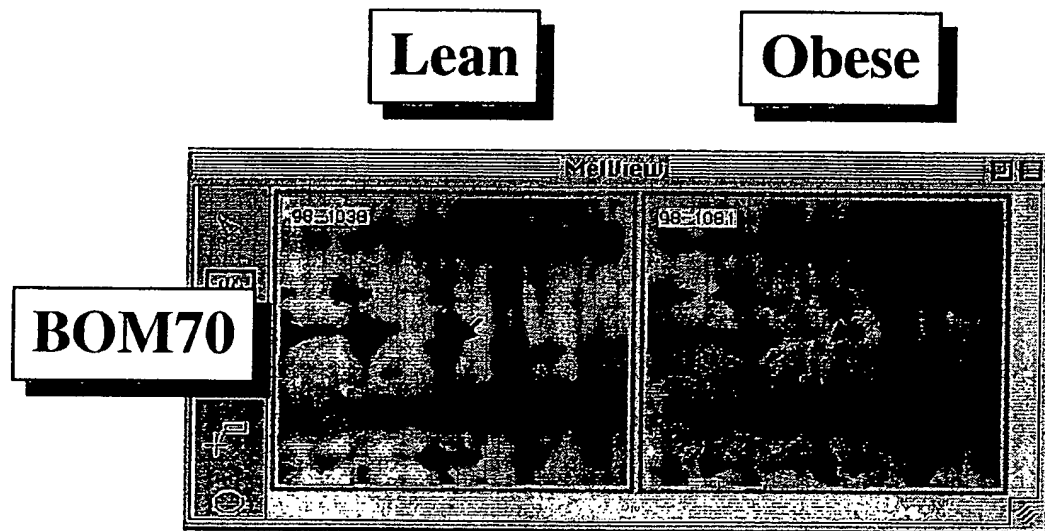
Figure 26

Figure 26 continued

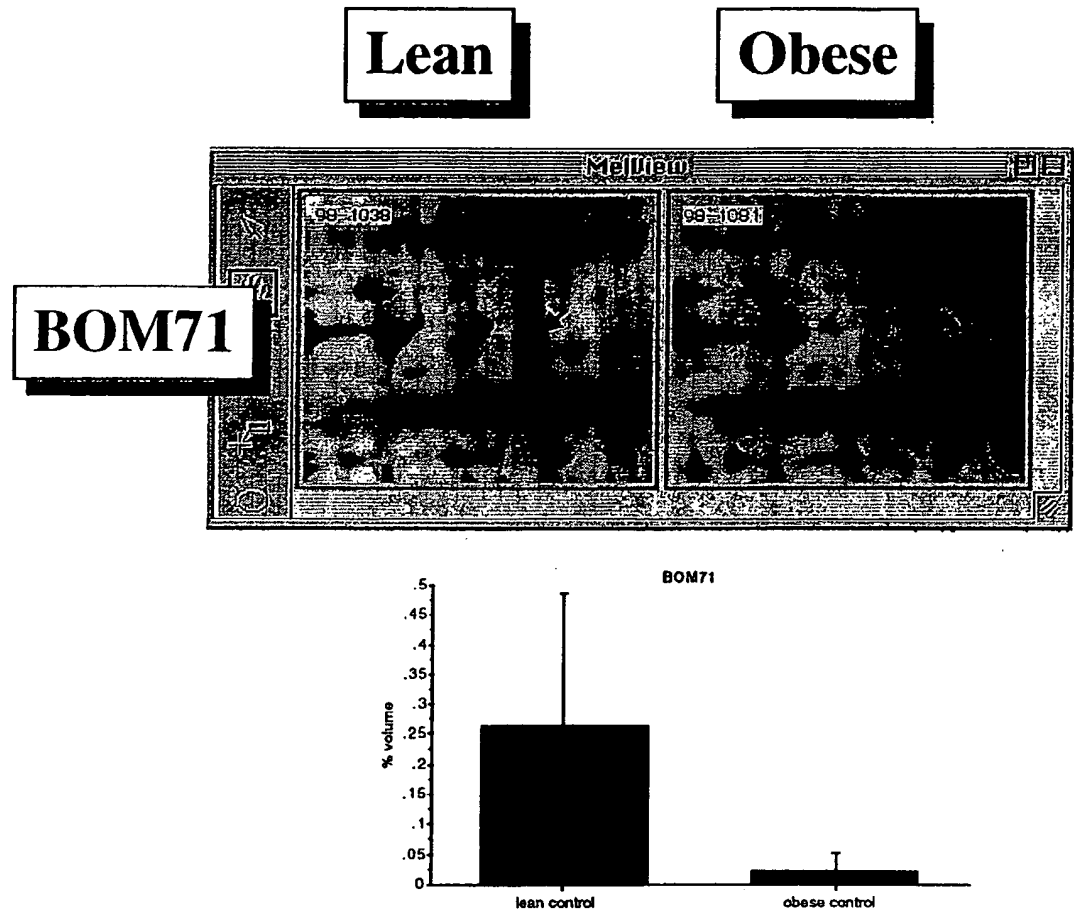


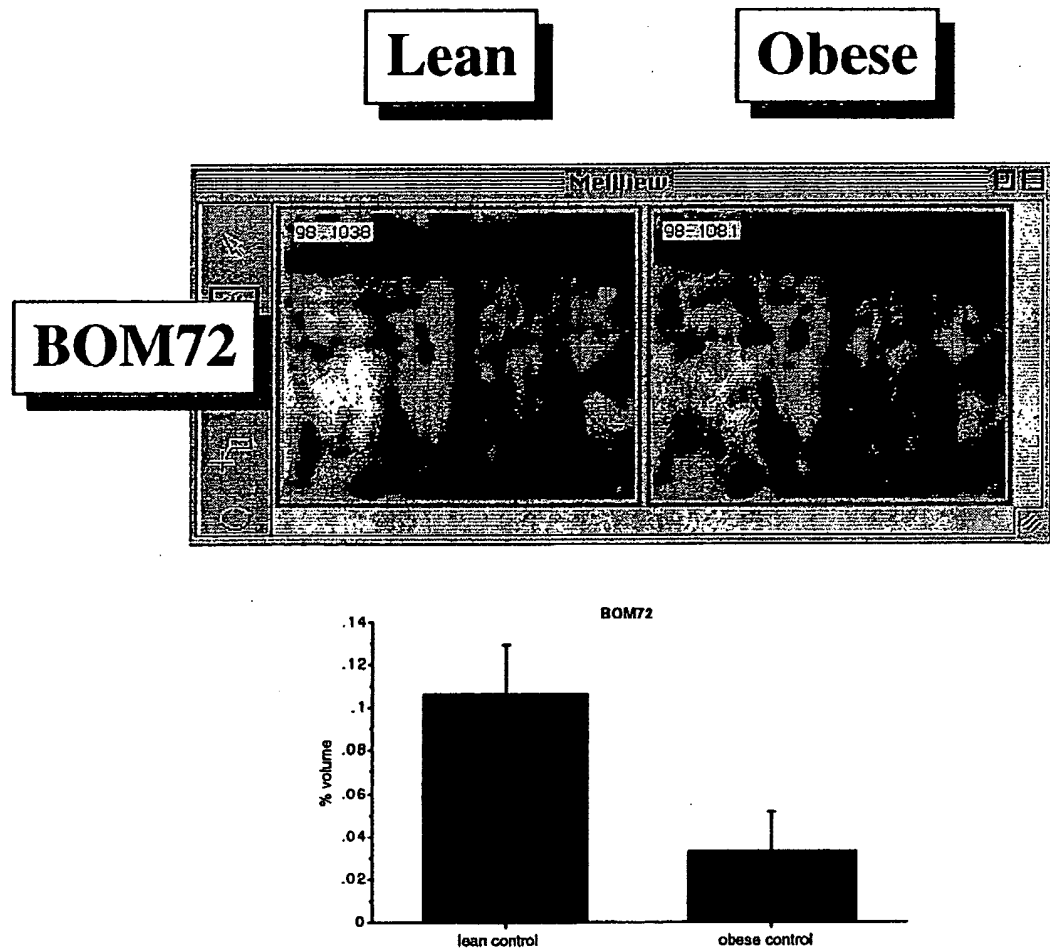
Figure 27

Figure 27 continued

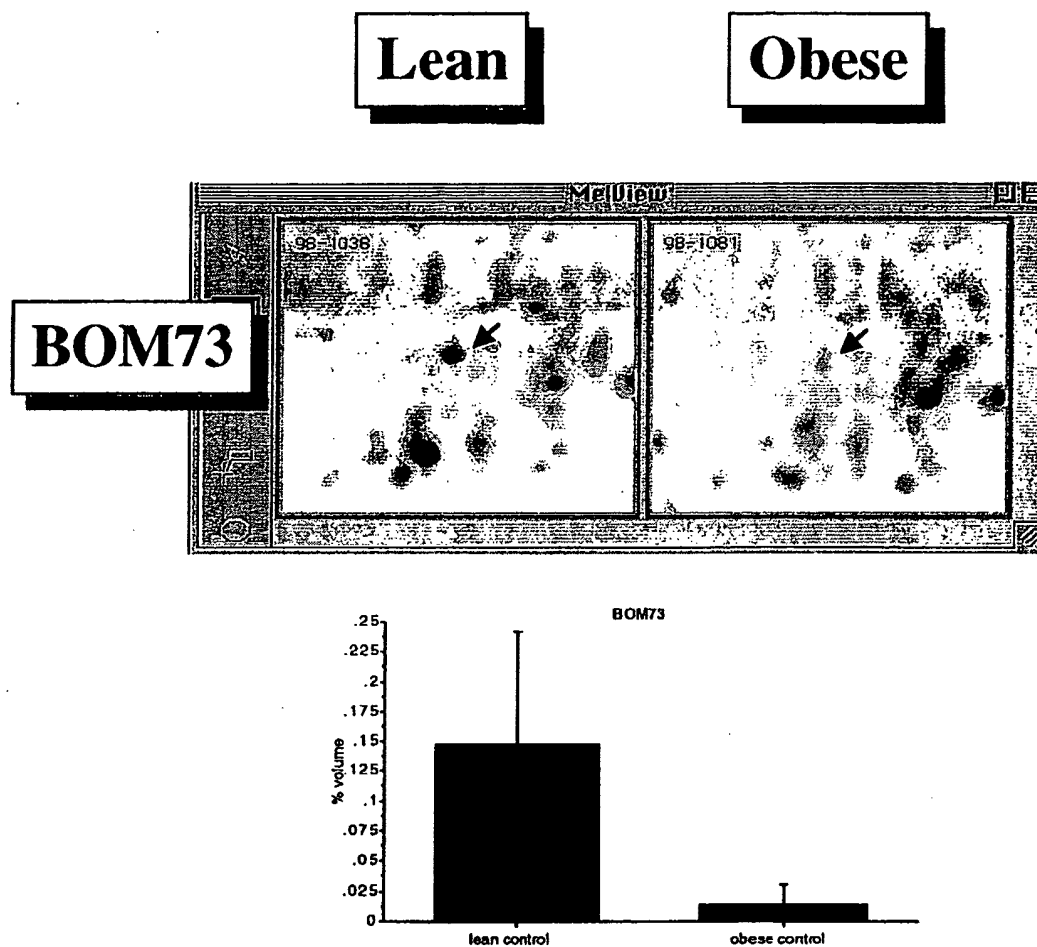


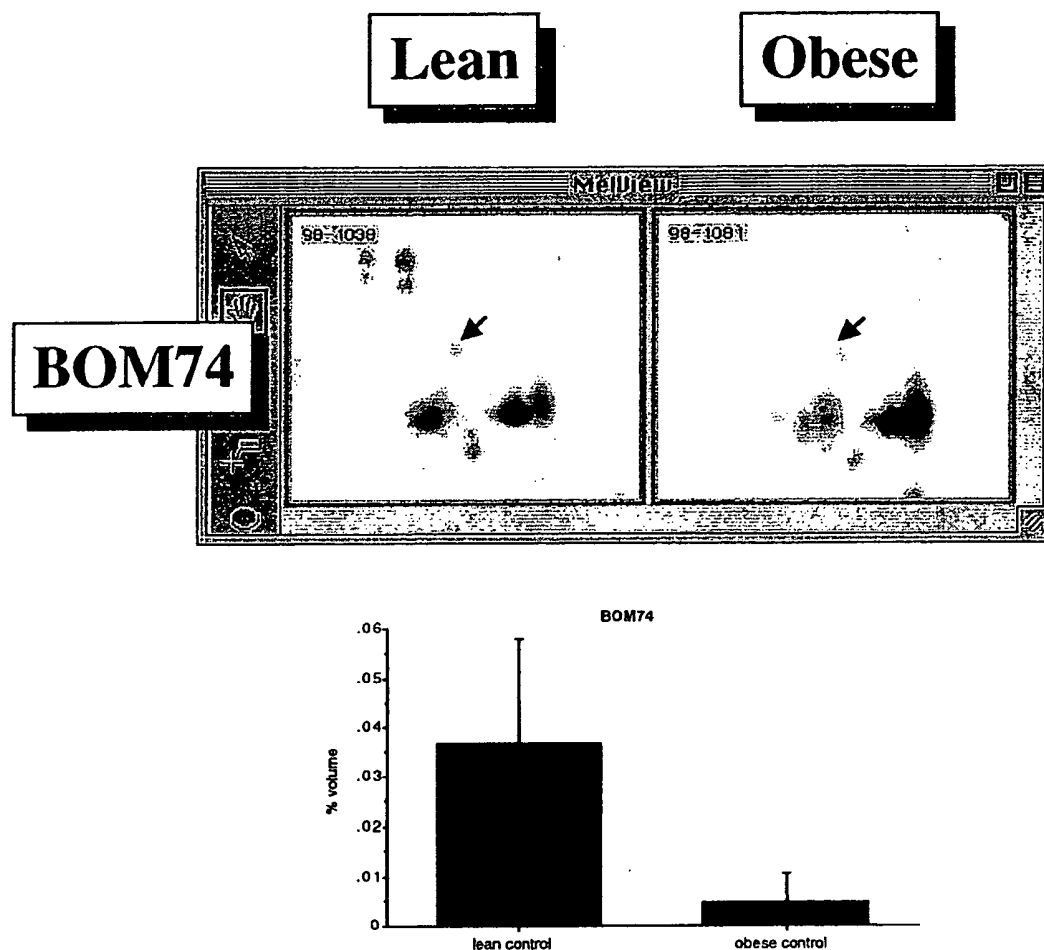
Figure 28

Figure 28 continued

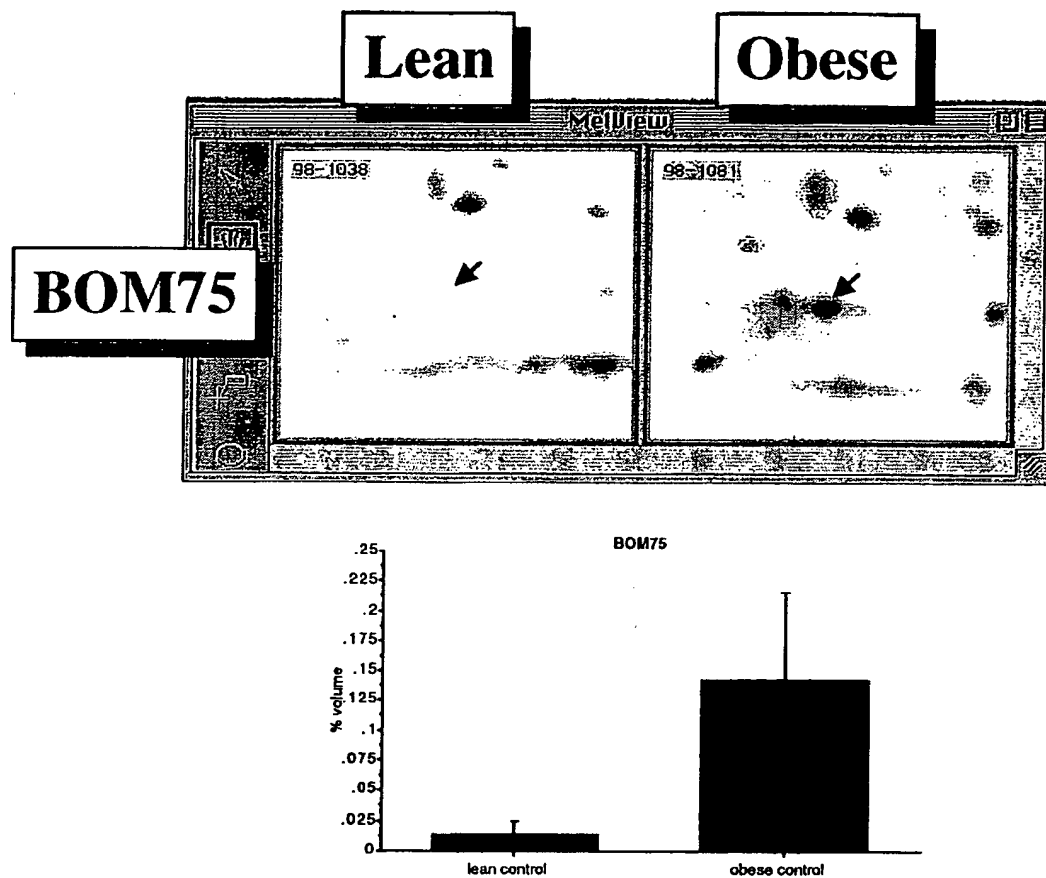


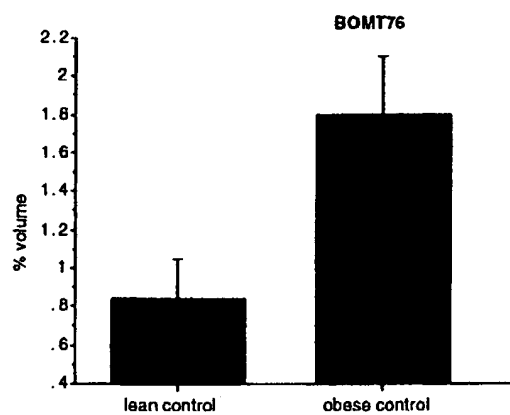
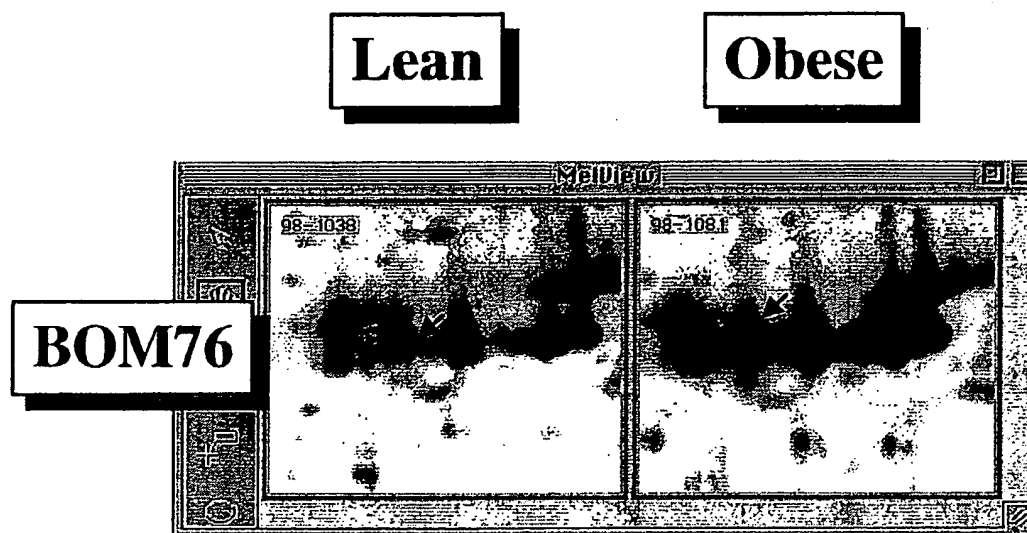
Figure 29

Figure 29 continued

